Vibrio cholerae non-01 and *V. mimicus* in diarrhoeal disease : a study of virulence factors

Thesis

UNRESTRICTED

Vibrio cholerae non-O1 and *V. mimicus* in diarrhoeal disease:

a study of virulence factors

Bengü Said

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Laboratory of Enteric Pathogens

Central Public Health Laboratory

61 Colindale Avenue

LONDON

NW9 5HT

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CONTAINS

PULLOUTS

" The Ganges, the sacred river that flows through Calcutta has become contaminated with a new deadly breed of cholera bacteria...*Vibrio cholerae* O139..."During the worst of it last month... we had 500 cholera cases arrive in one day, and our hospital has only 700 beds. We had to put them in the corridors, everywhere. They were four to a bed. I've never seen suffering on such a scale". " (McGirk 1993)

ABSTRACT

Vibrio cholerae non-O1 and *V. mimicus*, isolated from clinical and environmental sources, were examined for factors related to virulence. The aim was to identify factors which would distinguish pathogenic from non-pathogenic strains and to establish a correlation, if any, with serogroup. The lipopolysaccharide (LPS) of epidemic *V. cholerae* belonging to serogroups O1 and O139 are regarded as virulence factors. In this study certain other serogroups, such as O2, O5 and O9 were associated with diarrhoeal disease. LPS of these organisms may have a role in adhesion. Although 90% of *V. cholerae* non-O1 and *V. mimicus* colonised tissue culture cells, neither smooth LPS, the presence of flagella nor the possession of the toxin coregulated pilus gene (*tcpA*) were essential for adhesion. Cholera toxin (CT) is the factor responsible for the dramatic symptoms of epidemic cholera and is produced by *V. cholerae* serogroups O1 and O139. However, less than 1% of *V. cholerae* non-O1, non-O139 and *V. mimicus* possessed the gene for CT. Southern blot analysis of the CT genes revealed that most strains carried one CT gene except for serogroups O23 and O139 which had two hybridising fragments. The variations in fragment size within the O139 serogroup suggested that this serogroup was not strictly clonal. In addition to CT both *V. cholerae* non-O1 and *V. mimicus* expressed several other toxins, often concurrently, which made detection of specific toxins difficult. Over 80% of strains produced haemolysin(s) and cytotoxin(s). The cytotoxic effects on tissue culture cells masked cytotonic effects, such as that caused by CT. *V. cholerae* and *V. mimicus* did not produce verocytotoxin (VT) and did not possess the genes for VT. The heat-stable enterotoxin (NAG-ST) gene was found in 3% of *V. cholerae* non-O1 and 12% of *V. mimicus* strains. All *V. cholerae* strains belonging to serogroup O14, were NAG-ST positive and Southern blot analysis of the NAG-ST genes suggested that this serogroup represented a single clone. In addition to the established toxins two novel factors, which may contribute to the virulence of *V. cholerae* non-O1 and *V. mimicus*, were found. A novel cytotoxin, produced by 16% of strains, was expressed on Vero cells causing vacuolation of cell cytoplasm. Another putative "toxin" found in 19% of strains was demonstrated in

a GM1-ELISA. This "toxin" had the ability to bind ganglioside GM1 and shared antibody binding sites with CT. *V. cholerae* non-O1 and *V. mimicus* also expressed species specific high affinity iron chelating molecules; 74% of *V. cholerae* non-O1 produced enterochelin, whereas 90% of *V. mimicus* produced aerobactin. Aerobactin production is usually associated with invasive organisms and is unusual among *Vibrio spp.* None of the virulence factors, except CT, was more prevalent in clinical than in environmental strains. This is perhaps not surprising as vibrio-associated disease is often linked with exposure to the aquatic environment or consumption of seafood. Within *V. cholerae* the O1 and O139 serogroups were linked with CT and O14 with NAG-ST, in future, other serogroup - virulence factor associations may be found. For *V. mimicus* the main virulence factors expressed were aerobactin and NAG-ST. Therefore the pathogenicity of *V. cholerae* and *V. mimicus* appears to be multifactorial and it is likely that, as with diarrhoeagenic *E. coli*, a heterogeneous pattern of virulence will be found.

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I thank Dr. Bernard Rowe for allowing me to carry out this study and for his help and advice.

I dedicate this work to all my family,

especially to

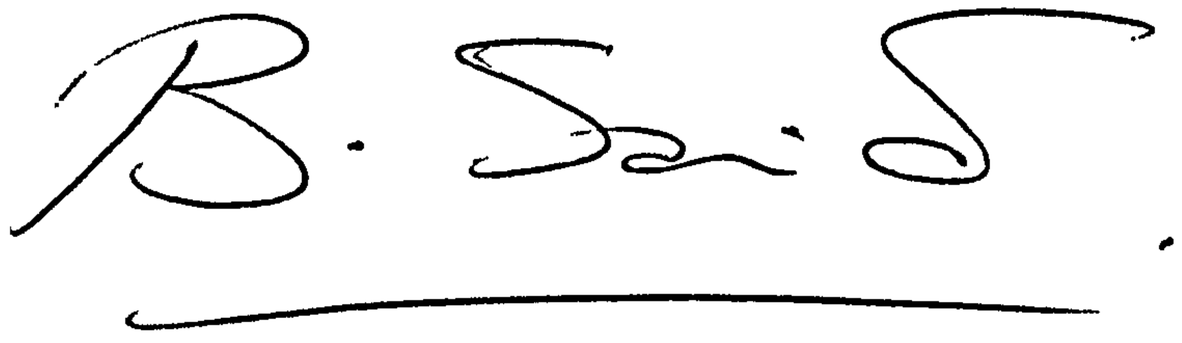
Graham and Ben

&

Anne ve Baba.

DECLARATION

I declare that the work presented here has been carried out by myself or where the assistance of other members of the Laboratory of Enteric Pathogens has been required, this fact has been acknowledged in the text of the thesis.



Bengü Said

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POSTGRADUATE RESEARCH STUDY COURSES

In partial fulfilment of the requirements of the degree, I have attended the following:

Postgraduate Research Methods Course (1987/1989) at the National Institute for Medical Research, Mill Hill, London, NW7 1AA;

Seminars in current research held at CPHL, London, NW9 5HT;

Practical Workshop in Immunological Techniques (1990) at Hatfield Polytechnic;

Radiological Workshop (1991) held at CPHL, London, NW9 5HT;

Molecular Microbiology Discussion Group, CPHL, London, NW9 5HT.

PRESENTATIONS AND PUBLISHED CONTRIBUTIONS

Said B. (1991). Production of cholera toxin (CT) and other enterotoxins by *Vibrio cholerae* non-O1 in relation to serogroup. PHLS 16th Annual Scientific Conference, Winchester.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BCL	Boehringer Corporation Limited
BCIP	4-bromo-chloro-indolyl phosphate
BHI	brain heart infusion broth
BSA	bovine serum albumin
bp	base pair
°C	degrees centigrade
Caco-2	human colonic carcinoma cells
cAMP	cyclic adenosine monophosphate
Cl^-	chloride ion
cm	centimetre
$CoCl_2$	cobalt chloride
CT	cholera toxin
CTAB	cetyltrimethyl ammonium bromide
CYE	casamino yeast extract broth
Dig-dUTP	digoxigenin-11-2'-deoxy-uridine-5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid, disodium salt
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
g	gram
g	relative centrifugal force
GM1	gangliosides of intestinal epithelial cells (galactosyl-*N*-acetyneuraminyl-[*N*-acetylneuraminyl]-galactosylglucosyl ceramide
h	hour
H	flagellar antigen
HeLa	Helen Lane cervical carcinoma cells
HEp-2	carcinoma of the larynx cells
Ig	immunoglobulin
HCl	Hydrochloric acid

kb	kilobase
kda	kilodalton
l	litre
LEP	Laboratory of Enteric Pathogens
LiCl	lithium chloride
LPS	lipopolysaccharide
LT	*E. coli* heat-labile enterotoxin
M	molar
Mda	megadalton
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
mls	millilitres
mM	millimolar
MOPS	morpholino propane sulphonic acid
MRHA	mannose resistant haemagglutination
MSHA	mannose sensitive haemagglutination
Na^+	sodium ion
NaCl	sodium chloride
NAD	nicotinamide adenosine diphosphate
NAG-ST	*V. cholerae* heat-stable enterotoxin
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
ng	nanogram
nm	nanometre
O	somatic antigen
O\129	pteridine compound (2,4 diamino-6,7 diisopropyl pteridine)
OD	absorbance units
OMP	Outer membrane protein
ONPG	o-nitrophenyl-ß-D-galactopyranoside
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PIH	passive immune haemolysis
PVP	polyvinyl pyrolidone
PW	peptone water
RNAase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SNAP	synthetic nucleic acid probe
SGB	syncase glucose broth
SSB	syncase sucrose broth
ST	*E. coli* heat-stable enterotoxin
TCM	tissue culture medium
TS	tris-succinate medium
Tris	tris (hydroxymethyl) aminomethane
TSB	trypticase soy broth
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet
V	volts
Vero	African green monkey kidney cells
VET-RPLA	*V. cholerae* enterotoxin - reversed passive latex agglutination
VP	Vogues-Proskauer test for acetylmethylcarbonil production
VT	verocytotoxin
v/v	volume to volume ratio
WHO	World Health Organisation
w/v	weight to volume ratio
Y1	mouse adrenal cortex tumour cells
%	percent

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INTRODUCTION

1.1 Identification, nomenclature and classification *Vibrio cholerae* and *V.mimicus*.

The family *Vibrionaceae* consists of four genera; *Vibrio, Aeromonas, Photobacterium* and *Plesiomonas*. The genus *Vibrio* includes several species which are pathogenic to humans, and infections are usually food-borne. Of more than 30 species, 11 are associated with human disease; *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. hollisiae*, *V. fluvialis*, *V. furnisii*, *V. vulnificus*, *V. damsela*, *V. metschnikovii* and *V. cincinnatienisis*. These divide biochemically into three groups (Table 1); *V.cholerae* and *V.mimicus* belong to group 1 (Furniss *et al.* 1978; Brayton *et al.* 1986; Janda *et al.* 1988).

V.cholerae and *V.mimicus* are short, rod-shaped (straight or slightly curved) gram-negative bacteria with a single polar flagellum. These organisms are facultatively anaerobic, oxidase positive and ferment glucose without forming gas. They grow readily on an agar medium at an optimal pH between 7.6 and 8.6. Colonies on agar are usually translucent and amorphous but sometimes wrinkled or rugose. Strains of *V. cholerae* and *V. mimicus* may produce a pellicle when grown in liquid media. Strains of *V. cholerae* and *V. mimicus* can grow in the absence of NaCl, a feature which distinguishes these bacteria from other vibrios. Other useful biochemical characteristics (Table 1) are; lysine decarboxylase (LDC) and ornithine decarboxylase (ODC) positive, but arginine dihydrolase (ADH) negative, acid from mannitol but not from inositol or arabinose.

The different serogroups of *V. cholerae* (Tables 2a and 2b) are very similar biochemically. However, all strains of *V. cholerae* serogroup O1 ferment mannose, whereas the reaction is variable with strains of *V. cholerae* belonging to non-O1 serogroups. *V. mimicus* is biochemically distinguished from *V. cholerae* by the inability to ferment sucrose, a negative reaction in the Vogues-Proskauer (VP) test, a lack of amylase production and failure to

Table 1: Biochemical characteristics of pathogenic *Vibrio spp.*

	NaCl -requiring	LDC	ODC	ADH	VP	Sucrose (gas)	Glucose
Group 1							
V. cholerae	-	+	+	-	v	+	-
V. mimicus	-	+	+	-	-	-	-
Group 2							
V. vulnificus	+	+	v	-	-	-	-
V. parahaemolyticus	+	+	+	-	-	-	-
V. alginolyticus	+	+	+	-	+	+	-
V. hollisae	+	-	-	-	-	+	-
V. cincinnatiensis	+	+	-	-	+	+	-
Group 3							
V. fluvialis	+	-	-	+	-	+	-
V. furnisii	+	-	-	+	-	+	+
V. damsela	+	v	+	v	v	-	-
*V. metschnikovii**	+	v	-	v	+	+	-

Group 1. Non-halophilic, arginine dihydrolase negative group.
Group 2. Halophilic, arginine dihydrolase negative group.
Group 3. Halophilic, arginine dihydrolase positive group.

**V.metschnikovii* is unusual in that it is the only member of the family which is oxidase negative.*

+ = positive, - = negative, v = variable reaction.

to haemagglutinate chick red blood cells (Sakazaki and Donovan 1984).

Individual strains of both species (*V. cholerae* and *V. mimicus*) can be differentiated by the structure of cell wall lipopolysaccharide, as detected by specific antisera prepared to the somatic ("O") antigen. Flagellar antigens are of little value for strain differentiation since flagellar or "H" antigens are seemingly identical for all strains of *V. cholerae* and *V. mimicus*. Early attempts to differentiate strains of *V. cholerae* by serology were performed by Gardner and Venkatraman (1935), who successfully divided a panel of strains of *V.cholerae* into six O-subgroups, in which O1 was assigned to 'cholera vibrios' (Gardner and Venkatraman 1935). In addition to the category 'cholera vibrio', a large number of vibrios, which are biochemically similar but which are not agglutinated by O1 antisera, have been recognised for many years in cholera-epidemic areas. These have been called non-agglutinable (NAG) or non-cholera vibrios (NCV). Since 1972, when the International Subcommittee on Taxonomy of Vibrios recommended that the species *V. cholerae* no longer be restricted to the cholera vibrio, they have been included in the species definition and are known collectively as *V. cholerae* non-O1 (Sakazaki and Donovan 1984; West and Colwell 1984). The antigenic scheme of Sakazaki and Donovan was extended throughout the 1970's, incorporating the scheme of Smith, to 83 serogroups (Table 2a) (Smith 1979; Sakazaki and Donovan 1984). Recent studies have further extended the scheme to show that the species *V. cholerae* contains over 140 serogroups (Table 2b) (Shimada *et al.* 1994).

Serogroup O1 can be divided into two biotypes, termed classical and El Tor. The classical biotype is differentiated from the El Tor by sensitivity to polymixin and to classical vibriophage IV. The El Tor biotype is sensitive to El Tor phage V and resistant to polymixin. In addition the El Tor biotype is usually haemolytic and can agglutinate chicken erythrocytes (Furniss *et al.* 1978). Both classical and El Tor

Table 2a: Reference strains for serogroups O1 - O83 of *V. cholerae*

O serogroup	Ref. strain	O serogroup	Ref. strain	O serogroup	Ref. strain
1 (Ogawa)	NCTC 10954/1	28	12530-62	56	475-75
1 (Inaba)	NCTC 8457/5	29	161-68	57	1463-76
2	NCTC 4711	30	12795-62	58	1162-74
3	NCTC 4715	31	5473-62	59	1333-74
4	NCTC 4716	32	171-68	60	195-75
5	B4202-64	33	151-68	61	12-74
6	7007-62	34	152-68	62	1-76
7	8394-62	35	1311-69	63	19-76
8	10317-62	36	1321-69	64	1280-75
9	112-68	37	1322-69	65	981-75
10	218-68	38	215-72	66	993-75
11	10843-62	39	225-68	67	121-79
12	211-72	40	212-72	68	293-78
13	1146-62	41	284-73	69	1861-79
14	B8645-64	42	104-73	70	1111-77
15	103-79	43	108-73	71	162-78
16	316-71	44	112-73	72	431-79
17	110-68	45	122-73	73	113-79
18	B5257-64	46	128-73	74	428-79
19	139-68	47	131-73	75	429-79
20	317-71	48	133-73	76	1158-76
21	109-68	49	1154-74	77	8-76
22	169-68	50	190-73	78	27-76
23	317-71	51	198-73	79	1103-76
24	14438-62	52	207-73	80	1421-77
25	14821-62	53	1157-74	81	318-78
26	334-72	54	1175-74	82	355-80
27	10432-62	55	197-75	83	1042-78

Table 2b: Reference strains for serogroups O84 - O140 of *V. cholerae*

O serogroup	Ref. strain	O serogroup	Ref. strain	O serogroup	Ref. strain
84	840-83	103	567-88	122	1133-80
85	1903-83	104	570-88	123	345-81
86	571-81	105	571-88	124	355-81
87	973-81	106	572-88	125	436-81
88	748-80	107	AU112	126	472-81
89	984-81	108	AU124	127	501-90
90	1457-78	109	AU165	128	819-87
91	796-80	110	AU256	129	420-81
92	NU193	111	AU291	130	447-81
93	S5046	112	AUR10	131	813-91
94	S5069	113	AU105	132	767-81
95	S5697	114	246-79	133	816-91
96	S5535	115	523-80	134	821-91
97	S7443	116	980-78	135	29-92
98	S6541	117	381-82	136	YCH11
99	554-88	118	58-91	137	448-92
100	558-88	119	353-81	138	455-92
101	559-88	120	686-91	139 (Bengal)	MO45
102	563-88	121	555-80	140 (Hakata)	487-85

biotypes can be further subdivided by serology into the Ogawa and Inaba subtypes; a third subtype, Hikojima, appears to be an unstable intermediary and is rarely seen (Sakazaki 1992). These subtypes are identified by O antigens designated a, b and c. Inaba contains somatic antigens a + c, Ogawa a + b and Hikojima a + b + c. The El Tor biotype, the cause of the seventh pandemic, is currently predominant in endemic and epidemic areas (Barrett and Blake 1981).

The reference strains of serogroups O20 (317-71), O71 (162-78), O101 (559-88), O114 (246-79), O115 (523-80), O116 (980-78), O117 (381-82), O135 (29-92) and O138 (455-92) are strains of *V. mimicus* (Table 2a and 2b). Since strains of *V. mimicus* and *V. cholerae* can belong to the same serogroups, a single scheme is applicable to both species.

1.2 Ecology and epidemiology

Strains of bacteria belonging to the genus *Vibrio* are free-living estuarine organisms, widely distributed throughout the world. Human pathogenic vibrios including *V. cholerae* and *V. mimicus* are naturally occurring in aquatic environments of areas free from endemic disease (Janda *et al.* 1988; West 1989; Morris 1990). The presence of these organisms is not always associated with faecal contamination or sporadic human infections (Feacham *et al.* 1981; Glass *et al.* 1983a; Miller *et al.* 1985), refuting the traditional concept that *V. cholerae* has a limited potential for survival outside the human intestine. The occurrence of infections with pathogenic vibrios in the United Kingdom is relatively infrequent compared with other developed countries such as the USA. Most cases of vibrio-associated diarrhoea occurring in the UK result from travellers returning from countries where cholera and cholera-like diseases are prevalent, although there may be under reporting due to lack of awareness of these pathogens. Cases of diarrhoea usually occur following ingestion of food exposed to a contaminated aquatic environment or the consumption of seafood. The occurrence of these pathogens and epidemiology of human infections caused by them is significantly influenced by

their microbial ecology.

Ecological studies indicate that water temperature, concentration of aquatic organic matter, salinity and the potential for association with sediments or the surfaces of higher organisms, significantly influence the occurrence and number of these pathogens in aquatic environments. Water temperature seems to be the single most important factor governing the incidence and density of pathogenic vibrios in natural aquatic environments. Seasonal and geographical variations occur which are dependent on water temperature (Roberts *et al.* 1982; West and Lee 1982; Williams and La Rock 1985; Nair *et al.* 1988). Most pathogenic Vibrios disappear from the pelagic environment at temperatures above 30°C and below 10°C but may persist in the aquatic sediments. Under favourable environmental conditions the vibrios can proliferate and re-emerge in the water (Hood and Ness 1982; West and Lee 1982; Williams and La Rock 1985).

Pathogenic Vibrios frequently occur in brackish coastal waters ranging in salinity from 5% to 30% NaCl (West and Lee 1982; Bockemuhl *et al.* 1986; Tison *et al.* 1986). In some freshwater areas (less than 5% salinity), pathogenic vibrios may survive due to the interaction of high water temperature and elevated organic nutrient concentration which overcomes the deleterious effect of low NaCl concentrations (DePaola *et al.* 1983; Rhodes *et al.* 1986; Nair *et al.* 1988). The prolonged survival of a toxigenic strain of *V. cholerae* O1 in low salinity and low nutrient environments has been demonstrated (Singleton *et al.* 1982a; Singleton *et al.* 1982b; Miller and Mekalanos 1984). These organisms also appear able to prolong their existence and maintain high numbers by association with plankton, shellfish and fish. In particular the chitin component in plankton appears to enhance significantly the phenomenon of prolonged survival (Huq *et al.* 1984). Bivalve molluscs have been shown to concentrate and harbour *V. cholerae* (DePaola *et al.* 1983). Storage of contaminated shellfish at inappropriate temperatures can lead to rapid proliferation of these bacteria within shellfish (Karunasagar *et al.* 1987). Marked seasonal variations in the presence of pathogenic vibrios in filter-feeding

shellfish have been described, and are thought to relate to the concentration of bacteria in the aquatic environment (Tison *et al.* 1986; Kelly and Dan Stroh 1988). Crustacean shellfish, such as crabs, shrimps and lobsters, can also become colonised; although this appears to depend on the concentration of bacteria and is more frequently observed at higher ambient temperatures (Davis and Sizemore 1982; Huq *et al.* 1986).

Both molluscs and crustaceans are implicated as vectors in food-poisoning incidents, with patients frequently having a history of consumption of seafood and shellfish. In the USA diarrhoeal disease is frequently associated with ingestion of raw shellfish, particularly oysters. In one study, strains of *V. cholerae* belonging to serogroup Smith O17 (now incorporated in the Sakazaki scheme and designated O2), accounted for 43% of the total number of strains of *V. cholerae* isolated (Morris *et al.* 1981).

Within areas where cholera is endemic, strains of *V. cholerae* harboured by animals are not considered as a significant cause of cholera (Sanyal *et al.* 1974; Miller *et al.* 1985); although, strains of *V. cholerae* non-O1 have been isolated from domestic animals, waterfowl and other wildlife in near-shore habitats of non-endemic cholera regions (DePaola 1981). In the UK, *V. cholerae* was not detected in the faeces of sheep grazing adjacent to ditchwater containing *V. cholerae* (Lee *et al.* 1982). In contrast, Rhodes *et al.* (Rhodes *et al.* 1985) reported *V. cholerae* non-O1 associated with enteric disease in horses, lambs and bison in Western Colorado where the organism occurs in some freshwater environments. Evidence is emerging to suggest that aquatic birds may serve as vectors for the dissemination of strains of *V. cholerae*. At a ditchwater site studied by Lee *et al.* (Lee *et al.* 1982) *V. cholerae* non-O1 was isolated from freshly voided droppings of swans nesting nearby. The study also reported a low frequency of intestinal carriage of *V. cholerae* non-O1 in seabirds caught in the UK. Ogg *et al.* (Ogg *et al.* 1989) isolated toxigenic strains of *V. cholerae* O1 and non-O1 from the gut of aquatic birds in central regions of the USA.

Laboratory studies have suggested another survival strategy which may be

adopted by vibrios; the so-called "viable but non-culturable" form. Under unfavourable conditions *V. cholerae* O1 can enter a state of dormancy (Xu *et al.* 1983; Roszak and Colwell 1987), with bacteria dividing normally when more favourable environmental conditions occur. This survival mechanism may be involved in the regular incidence of cholera epidemics in endemic areas, and explain how foci of *V. cholerae* persist in developed countries, such as around the Gulf of Mexico in the USA, even though conventional techniques show that *V. cholerae* is not continuously present. Although the survival of *V. cholerae* in fresh water is limited in laboratory experiments, studies in England, Australia and the USA (Desmarchelier and Reichelt 1981; Roberts *et al.* 1982; Lee *et al.* 1984) indicate that the organisms persist in natural environments such as rivers, lakes and estuaries.

Molecular biology techniques, such as chromosomal restriction endonuclease digest profiling, have established conclusively that natural aquatic environments are the epidemiological reservoir for toxigenic *V. cholerae* O1 in non-endemic cholera regions. In the Gulf of Mexico, a single strain of toxigenic O1 was responsible for a widely distributed series of cholera outbreaks over a decade (Kaper *et al.* 1982; Lin *et al.* 1986). CT genes were also found in a few strains of *V. cholerae* non-O1 from the same area (Kaper *et al.* 1986).

Strains of *V. cholerae* belonging to non-O1 serogroups occur more frequently and in higher numbers than strains belonging to the O1 serogroup, since O1 is but one of many serogroups which can be found in the aquatic environment (DePaola *et al.* 1983). Non-O1 serogroups may undergo bioconcentration in the aquatic environment in association with zooplankton and filter-feeding shellfish, similar to that described for serogroup O1 (Tison *et al.* 1986). In cases of diarrhoea caused by *V. cholerae* non-O1 the patients frequently have a history of consumption of seafood, especially oysters and crustaceans such as crabs, shrimps and lobsters. A study in the USA found a strong epidemiologic association with the consumption of raw oysters (Morris *et al.* 1981; Wilson *et al.* 1981). In a study in Cancun, Mexico 22 of 134 persons with diarrhoea, as well as sewage samples, untreated water samples

and raw seafood yielded *V. cholerae* non-O1 (Finch *et al.* 1987).

However, some infections caused by *V. cholerae* non-O1 do not appear to have a readily established link with aquatic environments (Thibaut *et al.* 1986; Safrin *et al.* 1988). It is therefore possible that other environmental niches exist and are yet to be fully identified. The capacity of *V. cholerae* non-O1 serogroups to survive and multiply in a wide range of foods appears to be greater than the capacity of *V. cholerae* O1 (Roberts and Gilbert 1979), suggesting that non-O1 serogroups may be transmitted by routes other than water or seafood in non-endemic areas. Humans may also act as carriers of *V. cholerae* as established in a study in India (Marwah *et al.* 1975). The majority of carriers were short term excreters of 1 to 7 days duration, with reports of an asymptomatic carriage rate as high as 9% in the 5 - 14 year old age group. In a study of Russian sailors it was found that the carrier state could last more than 60 days (Blake *et al.* 1980). However, there is no conclusive proof of spread by direct contact and the most important modes of spread are through food and water.

V. cholerae non-O1 can act as an opportunistic pathogen and host susceptibility may play an important role, especially in cases of extraintestinal illness where underlying disease (cirrhosis, leukaemia, malnutrition and gall bladder disease) is often present (Safrin *et al.* 1988).

1.3 *V. cholerae* and cholera

Diarrhoeal diseases are a significant cause of mortality and morbidity world-wide, particulary in developing countries. *V. cholerae* is an important cause of diarrhoea in both children and adults, particularly in Southern Asia and the Indian subcontinent where cholera and cholera-like diseases are endemic. Cholera is often associated with poverty, overcrowding and low socio-economic status.

In the last two centuries there have been seven cholera pandemics (Barua 1992). The first recorded cholera pandemic was in 1817; this and the following five pandemics were

attributed to strains of *V. cholerae* belonging to serogroup O1 biotype classical. The seventh pandemic was caused by *V. cholerae* O1 biotype El Tor. The epidemiology and mode of transmission of cholera was described by Snow after the "Broad Street pump" outbreak in 1849. The organism was discovered and named *V. cholerae* in 1854 by Pacini, and 30 years later, during the fifth pandemic, Robert Koch isolated and identified the causative agent of cholera (Barua 1992). When the El Tor biotype was discovered it was regarded as a harmless variant. However, *V. cholerae* El Tor strains rapidly displaced the classical biotype in endemic areas, and proved capable of causing epidemic cholera. The seventh and current pandemic started in 1961, and has spread from South East Asia to the Middle East and parts of Europe, reaching Africa in 1970 and South America in 1991 (Janda *et al.* 1988; Gangarosa and Tauxe 1992).

Worldwide the number of cholera cases reported to the World Health Organisation, in 1993, was 376,845 in 78 countries with 6,781 deaths (WHO 1994). This represents a substantial decrease in the figure for 1991, when due to the spread of the seventh pandemic to South America, 595,000 cases were reported (WHO 1994).

In Asia alone, there were 18,007 cases reported by 13 countries in 1989, this increased sharply in 1990, due to an outbreak in Nepal, to 30,979 cases reported by 12 countries (Anonymous 1991). In 1991, there were 49,791 cases and 1,286 deaths, this declined in 1992 to 16,299 cases and 372 deaths. Again the difference in numbers was due to the continuing outbreak in Nepal which accounted for 30,000 cases in 1991 (Anonymous 1993).

Transmission of *V. cholerae* is generally via the oral route, and food and water are the most common vectors. The minimum infective dose is 10^3 - 10^5 organisms, although stomach acidity influences significantly the dose required to cause overt disease (Levine *et al.* 1984; Drasar 1986). Strains of *V. cholerae* which produce cholera toxin (CT) are responsible for the disease cholera. In its most severe form (*cholera gravis*) victims produce large volumes (exceeding one litre per hour) of faeces termed "rice water" stools, resulting in severe dehydration causing poor skin turgor and sunken eyes. Stool losses generally peak during the

first 24 hours following the onset of symptoms and if patients are left untreated, they may develop circulatory collapse. However, only 2 to 11% of infections, caused by *V. cholerae* El Tor and classical biotypes respectively, develop the *cholera gravis* form of the disease (Barua and Burrows 1974). Fluid and electrolyte replacement is the main form of therapy, although antibiotics may shorten the duration of symptoms and excretion of the organism (Mahalanabis *et al.* 1992).

Strains not belonging to serogroup O1 (ie: *V. cholerae* non-O1) have been implicated in sporadic cases and outbreaks of diarrhoeal disease, sometimes indistinguishable from cholera (Blake *et al.* 1980). Strains of *V. cholerae* belonging to serogroups other than O1, produce CT only rarely and until 1993 had not been associated with epidemics of cholera; however, strains of *V. cholerae* belonging to serogroup O139 changed this trend.

1.4 Recent developments: the spread of *V.cholerae* O1 to South America and the emergence of *V. cholerae* O139

The seventh pandemic of cholera spread and increased steadily from 1961 until 1966, and in 1970 a major upsurge occurred as countries in Africa were infected. Reviewing the global cholera situation in 1980, the WHO working group expressed "concern that if cholera should reach any of the countries of South and Central America which are considered to be "receptive", there could be another dramatic increase in the number of affected countries" (WHO Scientific Working Group 1980). In January 1991, strains of *V. cholerae* O1 were isolated in Peru, the first time it had been identified in the Americas. By 1992 391,220 cases and 4002 deaths had been recorded (Anonymous 1992) in 14 of the 21 countries of South and Central America.

Untreated drinking water was thought to be a potential vehicle of infection with *V. cholerae* and the organism was also isolated from fish and seawater. The consumption of vegetables irrigated with raw waste water also contributed to the spread of this organism.

Genetic analysis revealed that strains isolated from the outbreak in South America were similar to strains of *V. cholerae* isolated from the pandemic occurring in Asia and Africa, and clearly distinguishable from strains causing sporadic cases of cholera along the Gulf coast of the USA (Wachsmuth *et al.* 1991; Wachsmuth *et al.* 1993).

As the seventh pandemic spread through South and Central America an apparently "new" strain of *V. cholerae* emerged in India and Bangladesh. The first reported outbreak of cholera caused by this organism occurred in October 1992 in Madras, India (Ramamurthy *et al.* 1993). The illness was clinically indistinguishable from epidemic cholera, except for the frequency of abdominal cramps (Bhattacharya *et al.* 1993). The outbreak spread to Calcutta (November 1992) with over 15,000 cases and 230 deaths. The number of cases of diarrhoea caused by *V. cholerae* O139 was increasing whereas the number of cases of cholera caused by *V. cholerae* O1 was decreasing; by December 1992 95% of cases were due to *V. cholerae* serogroup O139. There followed outbreaks in Southern Bangladesh (December 1992) (Albert *et al.* 1993), Dhaka (January and February 1993 - 10,000 cases, 500 deaths) and a further major outbreak in Calcutta (February 1993) (Anonymous 1993). The outbreaks, both in India and Bangladesh, were apparently caused by the same clone of *V. cholerae* O139 (Shimada *et al.* 1993). The severity of the illness, the high attack rate in the adult population and the lack of immunological protection in individuals with a history of clinical cholera suggested that this was a new strain, one to which the population had not been previously exposed. This was supported by the lack of protection conferred by vaccination with heat-inactivated whole cell *V. cholerae* O1 antigen. None of the strains reacted with a panel of 138 anti-lipopolysaccharide antisera prepared against the first 138 strains listed in Table 2a and 2b, and all were positive for CT. The outbreak strain was designated as *V. cholerae* serogroup O139, synonym Bengal, a novel non-O1 clone which appeared to be spreading and displacing the *V. cholerae* O1 El Tor strain (Swerdlow and Ries 1993) just as the El Tor strain once superseded the classical *V. cholerae* O1. Sporadic indigenous cases were also confirmed from Thailand, China, Malaysia

and Nepal (WHO 1994); imported cases were reported in Estonia, Germany, USA and UK (Anonymous 1993; Cheasty *et al.* 1993). This strain of *V. cholerae* belonging to serogroup O139 may have the potential to cause a new pandemic (Swerdlow and Ries 1993).

Despite microbiological and clinical advances, cholera remains a persistent problem for the developing and to a lesser extent, the developed world. The emergence of strains of *V. cholerae* belonging to serogroup O139, the appearance of multiply resistant strains of *V. cholerae* and the spread of the 7th pandemic to South America has lead to renewed interest in *V. cholerae*.

1.5 *V. cholerae* non-O1, *V. mimicus* and associated clinical disease

Serology has enabled strains of *V. cholerae* and *V. mimicus* to be divided into 140 groups based on epitopes located on *V. cholerae* lipopolysaccharide, also termed the somatic or "O" antigens. Strains belonging to *V. cholerae* serogroup O1 regardless of biotype are agglutinated by anti-O1 antisera; while all other serogroups, which are not agglutinated by anti-O1 antisera, are designated *V. cholerae* non-O1. Strains of *V. mimicus*, once thought to be a variant of *V. cholerae* unable to utilize sucrose, have now been classified as a distinct species (Davis *et al.* 1981).

Strains of *V. cholerae* belonging to serogroups other than O1 have been implicated in sporadic cases and outbreaks of gastrointestinal disease. The symptoms vary from bloody diarrhoea with fever to a severe watery diarrhoea indistinguishable from cholera. In 1986 it was reported that strains of *V. cholerae* belonging to serogroups other than O1 were a significant cause of mortality in Bangladesh with 25.8% fatality (Islam and Shahid 1986). Volunteer studies in 1990 (Morris 1990) confirmed that strains of *V. cholerae* non-O1 can cause diarrhoea with a severity comparable to that seen in cholera even by strains unable to make CT, a key virulence property expressed by *V. cholerae* O1. If CT is produced by non-O1 serogroups, as with the epidemic *V. cholerae* O139 strain, the presentation is typical of clinical

cholera. Reports of outbreaks possibly caused by strains of *V. cholerae* other than O1 date back to 1954 (Yajnik and Prasad 1954); since then there have been several published reports of outbreaks of gastroenteritis attributable to strains of *V. cholerae* non-O1 and some of these have, retrospectively, been linked to particular O-serogroups (Donovan 1984). For example, during an outbreak of cholera in Czechoslovakia (1965) there were 56 cases, and *V. cholerae* was isolated from 42 of the patients. Strains of *V. cholerae* belonging to serogroup O5 were implicated, with potato salad as the probable vehicle of transmission (Aldova *et al.* 1968) (Table 3). Another outbreak occurred among passengers onboard an aeroplane destined for Australia (1973); the outbreak was linked to the consumption of chopped egg but the serogroup of *V. cholerae* involved was not identified (Dakin *et al.* 1974). In a water-borne outbreak, reported in 1971, there were 544 cases and 31 fatalities. The outbreak was suspected to have resulted from faecal contamination of drinking water and caused by a strain of *V. cholerae* serogroup O37 (Kamal 1971).

Sporadic cases associated with *V. cholerae* non-O1 diarrhoea have also been reported. McIntyre reported 19 persons with sporadic illness from Bangladesh; many had severe dehydrating diarrhoea and 50% required intravenous fluid therapy (McIntyre *et al.* 1965). Spira *et al.* reported on 14 adults with cholera in Bangladesh, symptoms included vomiting (100%), abdominal pain (71%), pyrexia (43%) and muscle cramps (21%). The mean duration of illness was 42 hours (Spira *et al.* 1978). Another study from the USA reported on 13 patients whose symptoms included abdominal cramps (92%), nausea (77%), vomiting (69%), chills (64%), fever (58%), mucus in stools (30%), and bloody stools (8%); the duration was approximately 7 days (Hughes *et al.* 1978). In summary, a range of symptoms has been associated with cases of *V. cholerae* non-O1 gastroenteritis, including fever and bloody diarrhoea which are encountered only rarely in "typical" cases of cholera.

Table 3: *V. cholerae* non-O1 outbreaks

Year	Place	Clinical details	Suspected mode of transmission	Serogroup	Virulence factor	Reference
1965	Czechoslovakia	Diarrhoea 1-2 days vomiting(25%) fever(11%)	Potato salad	O5	?	Aldova
1968	Sudan	Rice water stools	Water	O37	?CT	Kamal
1974	Australia	Diarrhoea & vomiting abdominal cramps 18-24h	Chopped egg	?	?	Dakin
1992	India	Acute cholera-like diarrhoea	?Water	O139	CT	Ramamurthy
1992	Bangladesh	Acute cholera-like diarrhoea	?Water	O139	CT	Albert

V. cholerae has been established as the etiologic agent of cholera and until recently it was generally accepted that only strains of *V. cholerae* belonging to serogroup O1 were responsible for causing epidemics of cholera. However, in 1992/3 a non-O1 strain of *V. cholerae* emerged with epidemic, and possibly pandemic potential (see section 1.4). This strain of *V. cholerae* belonging to O139 appeared to be displacing strains of *V. cholerae* belonging to serotype O1 El Tor in endemic areas, and may prove to be the cause of an eighth pandemic, the first caused by a non-O1 strain of *V. cholerae* (Swerdlow and Ries 1993).

Strains of *V. cholerae* belonging to serogroups O1 and O139 produce cholera toxin, however toxin production is not a prerequisite for infection and non-toxigenic strains of *V. cholerae* O1 are able to cause illness. This usually involves diarrhoea, and symptoms vary from the mild and transitory to an illness resembling *cholera gravis*. Infections with non-toxigenic *V. cholerae* O1 may be of increasing prevalence (Honda *et al.* 1988). Most *V. cholerae* non-O1 do not produce CT, although other enterotoxigenic mechanisms may or may not be present.

V. mimicus has also been reported as a causative agent of diarrhoea (Shandera *et al.* 1983); however, as with *V. cholerae* non-O1, production of CT is rare and most strains produce toxins other than CT (Chowdhury *et al.* 1987; Ramamurthy *et al.* 1994). The wider range of presenting symptoms seen with *V. mimicus* and *V. cholerae* non-O1 as opposed to *V. cholerae* O1 infections is probably related to the presence or absence of a number of virulence factors.

Strains of both *V. cholerae* non-O1 and *V. mimicus* are also associated with non-gastrointestinal infections and are capable of causing extraintestinal infections. Septicaemia may occur, with the causative organism being present in bile, gall bladder, urine, blood, sputum, appendix, peritoneal fluid and cerebrospinal fluid (Safrin *et al.* 1988; Clark *et al.* 1989). In general, patients which develop septicaemia have underlying disease, such as cirrhosis of the liver, or have wound infections contaminated with sea or estuary water.

Extraintestinal infections, due to *V. cholerae* O1, have also been reported, although these are rare (Johnston *et al.* 1983). Where extraintestinal infections have occurred the strains of *V. cholerae* were found not to produce CT indicating other factors may be involved. The pathogenic mechanisms possessed by strains of *V. cholerae* causing extra-intestinal infections remain to be elucidated.

1.6 Pathogenic mechanisms and virulence

Pathogenicity describes the ability of an organism to produce disease, and pathogenic bacteria may show variation in pathogenicity relating to the virulence of a given organism. In order to produce disease a pathogen must be able to complete a defined disease process (Smith 1978).

The pathogenesis of most diseases generally involves one or more of the following four steps, as described by Smith (1978).

(1) Entry into a host, and colonisation of mucous membranes.

(2) Multiplication in the physical and chemical conditions of the host environment.

(3) Avoidance of the action of humoral (in body fluids) and cellular host defense mechanisms.

(4) Biochemical processes resulting in host tissue damage and possibly fatal effects associated with the disease.

The microbial factors responsible for these steps in disease production are virulence determinants or virulence factors. For an organism to cause disease, several different factors may be required to accomplish the full pathogenesis of disease.

Strains of *V. cholerae* and *V. mimicus* are usually ingested in contaminated food

or water. Once inside the host the organisms must resist gastric acid, enzymes and bile, in this context the presence of food or $NaHCO_3$ is necessary for the organisms to survive. A prelude to infection involves the penetration of the gut mucous layer, the ability to resist peristalsis and compete favourably with commensal gut micro-organisms (for example, lactobacilli which are inhibitory to enteric pathogens) (Levine *et al.* 1984). Properties such as motility, the production of mucinases and other proteolytic enzymes enable the bacteria to penetrate intestinal mucous and to reach enterocytes directed by a chemotactic response. Strains of *V. cholerae* attach preferentially to the epithelium of the upper rather than the lower bowel.

Other recognised bacterial pathogenic properties include long-chain lipopolysaccharide, capsules and extra-cellular protein layers which enable organisms to attach to the epithelium or resist host defences; however, for strains of *V. cholerae*, these factors have not been investigated fully. Following initial adherence, organisms may proliferate on the epithelial surface, and may remain associated with the gut mucosa (for example *Shigella dysenteriae*) or may traverse the intestinal wall and enter the host circulation (for example *Salmonella typhi*).

Regardless of whether pathogenic bacteria are non-invasive or extra-intestinal, additional pathogenic mechanisms are usually involved in pathogenesis. Although the manifestations of disease may result from the release of various "aggressins" (Smith 1978), a major pathogenic mechanism is the ability to produce toxins. A powerful heat-labile exotoxin, cholera toxin or CT is the main virulence factor associated with strains of *V. cholerae* belonging to serogroups O1 and O139 which cause epidemic cholera. However, strains of *V. cholerae* which do not produce CT have been isolated from patients with diarrhoeal disease (McIntyre *et al.* 1965; Morris *et al.* 1984; Batchelor and Wignall 1988) and a volunteer study has shown that *V. cholerae* non-O1 which do not possess the CT gene can elicit diarrhoea with a severity comparable to cholera (Morris *et al.* 1981). "Non-toxigenic" (CT negative) strains of *V. cholerae* O1 have also been reported, for example a "non-toxigenic" strain was isolated

from a patient with severe gastroenteritis after consuming oysters (Morris *et al.* 1984). These so-called non-toxigenic strains may in fact be producing a toxin distinct from CT. Saha and Sanyal (1988) found evidence of the production, by an environmental *V. cholerae*, of a second enterotoxin, which was different from CT in antigenic nature, receptor site, mode of action and genetic homology.

Strains of *V. cholerae* belonging to serogroups other than O1 and O139 appear to cause diarrhoeal disease by mechanisms other than production of CT (Honda and Miwatani 1988). The wide range of extracellular toxins elaborated by *V. cholerae* non-O1 strains include: CT and cholera-like toxin (Shankar *et al.* 1982; Yamamoto *et al.* 1983a; Yamamoto *et al.* 1983b), heat-stable enterotoxin (NAG-ST) (Takao *et al.* 1985; Arita *et al.* 1986), cytotoxins (Janda *et al.* 1988), a specific cytotoxin resembling Shiga-like toxin (O'Brien *et al.* 1984), and haemolysins (Nishibuchi and Seidler 1983; Datta-Roy *et al.* 1986) including a thermostable haemolysin related to the Thermostable direct haemolysin (TDH) of *V. parahaemolyticus* (Honda *et al.* 1985; Yoh *et al.* 1986). The pathogenicity of *V. mimicus* appears to be similar to *V. cholerae* non-O1. Production of; CT (Spira and Fedorka-Cray 1984; Chowdhury *et al.* 1987), ST (Arita *et al.* 1991), cytotoxins (Ramamurthy *et al.* 1994) and TDH (Nishibuchi *et al.* 1990) by strains of *V. mimicus* have been described.

Of the toxins known to be expressed by strains of *V. cholerae*, not all are expressed by all strains, although certain strains may produce more than one toxin. Bhattacharya *et al.* (Bhattacharya *et al.* 1992) reported a strain of *V. cholerae* belonging to serogroup O5 which caused severe watery diarrhoea and dehydration in an Australian tourist, who required hospitalisation. This strain was negative for CT and NAG-ST but produced a haemolysin and a cytotoxin, suggesting that these toxins may be involved in pathogenesis. Strains of *V. mimicus* producing more than one toxin have also been reported (Ramamurthy *et al.* 1994). There appears to be geographic variability in the frequency with which strains produce certain toxins. Production of CT is rare among clinical isolates in the USA (Morris *et*

al. 1981; Roberts *et al.* 1982), but has been described in 30-40% of clinical isolates from India and Bangladesh (Blake *et al.* 1980; Datta-Roy *et al.* 1986).

A variety of animal models has been used to assess the production of toxins. They include rabbit ligated intestinal loop (ileal loop, De test), infant rabbit (Dutta test) and infant mouse assays (Ichinose *et al.* 1987). Even when cell-free supernatants have failed to produce detectable enterotoxin-like activity, the ability of live cultures to produce fluid accumulation in rabbit ileal loops has been demonstrated with *V. cholerae* non-O1 (Madden *et al.* 1981; Simpson *et al.* 1987). Cell tests and immunological tests can also be used to detect some toxins. Optimal cultural conditions for toxin production can vary from strain to strain as toxin production is affected by the culture medium used, pH, requirement for NaCl, aeration versus static incubation and addition of lincomycin (Yamamoto *et al.* 1981; Nishibuchi and Seidler 1983; Turnbull *et al.* 1985). Tissue culture methods using Y1 mouse adrenal cells or Chinese hamster ovary cells are generally used to detect the closely related heat-labile enterotoxins LT and CT (Donta *et al.* 1974; Guerrant *et al.* 1974). Although tissue culture tests are sensitive and relatively easy to perform, they require cell maintenance and the proteases/ haemolysins of some strains may mask the detection of cytotonic enterotoxin activity. Where sera are available enzyme linked immunosorbent assays (ELISA) and latex agglutination tests can be used but they may detect inactive, partial toxin (choleragenoid) as opposed to whole toxin. Gene probes are specific, as well as sensitive, and now that non-radioactive labelling methods are available the only drawback may be that the gene detected is inactive. In addition to toxin production *V. cholerae* and *V. mimicus* may possess a number of other virulence determinants, including: cell-associated haemagglutinins, flagella and fimbriae, a range of outer membrane proteins and high affinity iron uptake systems. The precise role of many of these factors in disease is not known (Robins-Browne *et al.* 1977; Shehabi and Richardson 1985; West 1989; Ramamurthy *et al.* 1994). The most extensively studied virulence factor produced by strains of *V. cholerae* is the ability to produce a potent exotoxin, CT.

1.6.1 Cholera Toxin (CT) and related heat-labile toxins

The characteristic symptoms of cholera result from the action of a single toxin. The production of this powerful exotoxin was first shown by De *et al.* (De and Chatterjee 1953; De 1959); a ligated segment of small intestine in a living rabbit filled with fluid following intraluminal injection of *V. cholerae* or sterile culture filtrates. The toxin was purified in 1969 by Finkelstein (Finkelstein and Lo Spalluto 1969). The mode of action of CT was first suggested by the work of Field *et al.* who measured the potential difference across stripped segments of ileal mucosa using an Ussing chamber (Field *et al.* 1971). They found that the addition of cyclic adenosine monophosphate (cAMP) caused the direction of chloride ion (Cl^-) transport to be reversed, so that there was active secretion of Cl^- from the mucosal surface. Absorption of sodium ions (Na^+) was also reduced. The net result of such changes in ion balance was a passive efflux of water from the mucosal surface of the tissue. Under the same conditions sterile culture filtrates of *V.cholerae* mimicked the effects of cAMP thus causing diarrhoea. Using purified CT it was shown that CT stimulates adenylate cyclase activity in the intestinal tract, leading to increased levels of cAMP and thereby altering ion transport at the mucosal surface (Stephen and Pietrowski 1981).

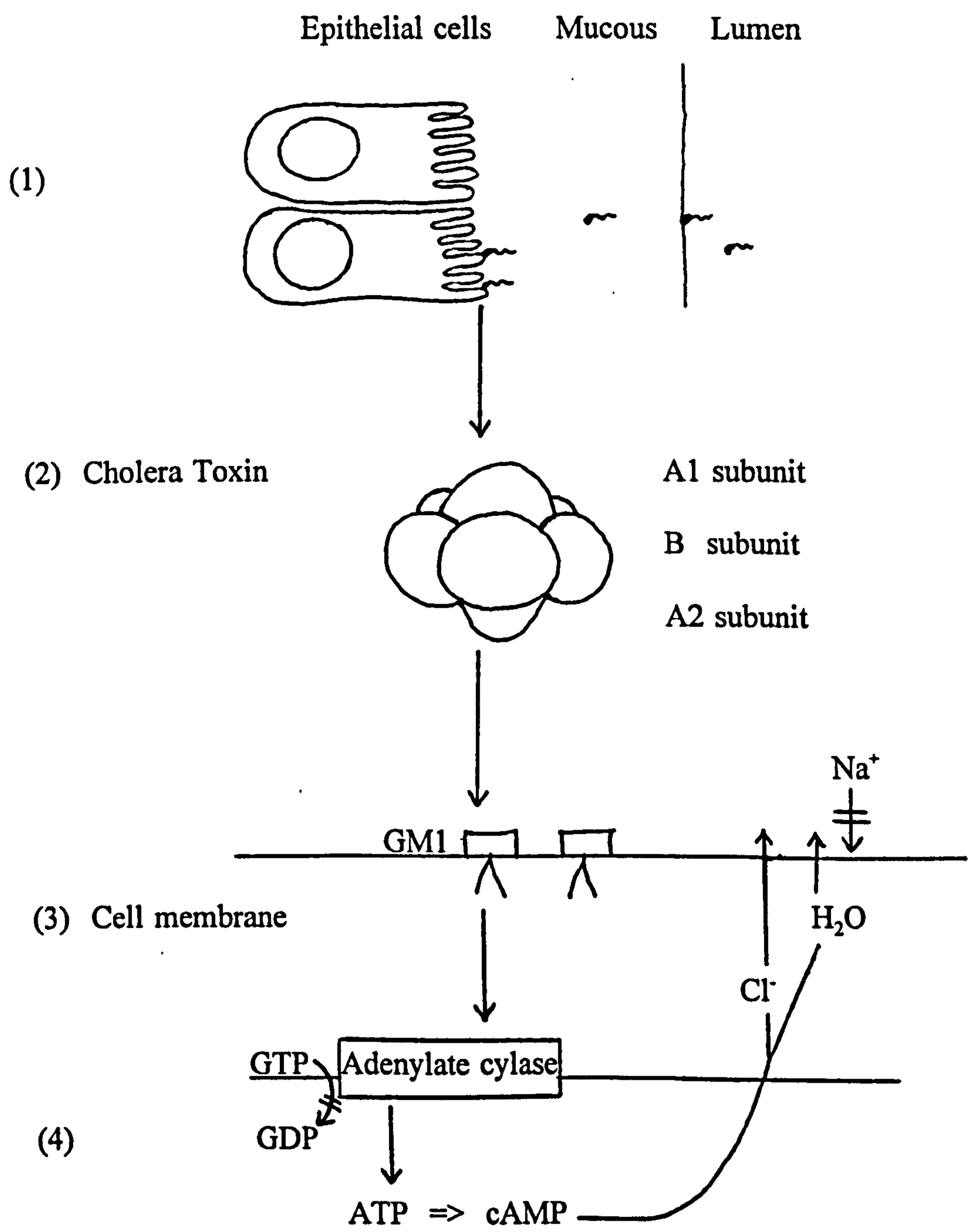
CT is a protein toxin consisting of two subunits: a single A (active) subunit of molecular weight 28,000 which is covalently attached to a 58,000 aggregate of five B (binding) subunits, each with a molecular weight of 11,600 (Holmgren 1981). The five B subunits form a stable ring pentamer structure. The A subunit is linked to and partially inserted into the B ring. The B subunit is responsible for specific high affinity binding of the toxin to the GM1 gangliosides of intestinal epithelial cells (Holmgren *et al.* 1973; van Heyningen *et al.* 1974; Svennerholm and Holmgren 1978). The A subunit is synthesised as a single polypeptide chain and penetrates the cell membrane to initiate activation of adenylate cyclase. Once penetration has occurred the A subunit is split into fragments, A1 (22,500) and A2 (5,500), by "nicking" between two cysteine residues. This releases the A1 fragment from the A2 portion which

attaches it to the B pentamer (Gill 1976). The reduction of the disulphide bond between the A1 and A2 fragments appears to be necessary for the activity of the toxin on adenylate cyclase. The activation depends on NAD, undefined cellular cytosol factors, and ATP, in addition to the A1 fragment of CT and the cell membrane (Gill and King 1975). CT has ADP-ribosyltransferase activity and catalyses the reaction: NAD + acceptor protein -> ADP-ribose-acceptor protein + nicotinamide + H^+ (Moss and Vaughan 1977). The acceptor protein is the guanylnucleotide-binding component (the G_s protein) of the membrane-bound adenylate cyclase (Pfeuffer and Cassel 1978). Adenylate cyclase is active while GTP is bound to the G_s protein but becomes inactive when GTP is hydrolysed to GDP. CT, therefore, stabilises adenylate cyclase in an active conformation (Figure 1).

CT is closely linked with *V. cholerae* serogroups O1 and O139 which cause epidemic cholera. The pathogenicity of *V. cholerae* O1 has been comprehensively reviewed (Levine *et al.* 1984; Mekalanos *et al.* 1988; Holmgren 1992). Other *V. cholerae* serogroups also possess the CT gene and produce CT. CT production in strains of *V. cholerae* non-O1 has been reported from Bangladesh and India; Datta-Roy (Datta-Roy *et al.* 1986) found an incidence of 26% (9/34) in clinical isolates and 10% (1/10) in environmental isolates. The incidence in other parts of the world is lower. A study in Thailand (Hanchalay *et al.* 1985) found none of 44 human isolates and 2% (5/237) of environmental isolates were CT positive. In a study of environmental isolates from the Louisiana Gulf coast (Roberts *et al.* 1982) it was found that only 0.3% (7/2500) produced CT. A study of 790 *V. cholerae* non-O1 strains from 50 countries, isolated from both environmental and human sources also found a low incidence (0.25%) of the CT gene (Said *et al.* 1994).

However, cholera-like diarrhoea has been associated with CT-negative strains of both *V. cholerae* O1 and non-O1 (Spira and Fedorka-Cray 1983; Spira *et al.* 1986). Production of a CT-like toxin was first suggested by Zinnaka & Carpenter (Zinnaka and Carpenter 1972) and Ohashi *et al.* (Ohashi *et al.* 1972). CT-like toxins have been found in some strains of

Figure 1: Mechanism by which cholera causes diarrhoea.



(1) Highly motile cholera vibrios propel themselves through the mucous layer and adhere to the epithelial cells.
(2) As the organisms multiply CT is produced and exported out of the periplasm; CT binds to the GM1 ganglioside receptors on the surface of the cell membrane.
(3) The A1 fragment interacts with the adenylate cyclase complex, located on the basal membrane, inhibiting the hydrolysis of GTP -> GDP.
(4) The adenylate cyclase complex remains active and stimulates cAMP causing the efflux of Cl^- and H_2O.

V. cholerae non-O1 and *V. mimicus* (Shankar *et al.* 1982; Spira and Fedorka-Cray 1983; Yamamoto *et al.* 1983b; Spira and Fedorka-Cray 1984; Yamamoto *et al.* 1985; Spira *et al.* 1986). The ability to produce CT-like toxin appears to be clinically relevant (Zinnaka and Carpenter 1972; Zinnaka *et al.* 1973; Spira and Daniel 1979).

CT and the heat-labile enterotoxin (LT) of *Escherichia coli* share similar modes of action, common antigenic determinants and possess extensive homology of amino acid sequence (Holmgren *et al.* 1973; Gill and Rappaport 1979). CT and LT are part of a heterogeneous family of enterotoxins and different forms of LT have been described ie: LTh-I, LTp-I, LTIIa and LTIIb (Guth *et al.* 1986). Similarly different forms of CT may exist with differences in molecular structure between enterotoxins produced by different serogroups of *V. cholerae* (Finkelstein *et al.* 1987).

A variety of techniques including antigenic similarity, biologic activity and electrophoretic mobility in gels have been used to detect these toxins. Homologous or closely related gene sequences have been detected by hybridisation to the CT and LT probes (Moseley and Falkow 1980; Kaper *et al.* 1981). Yamamoto and colleagues found toxin purified from *V. cholerae* non-O1 from Louisiana was indistinguishable from cholera toxin, but that another *V. cholerae* non-O1, from a patient with diarrhoea, produced a toxin biologically and immunologically similar but not identical to CT in molecular structure (Yamamoto *et al.* 1983b). Spira & Fedorka-Cray reported CT-like toxin from *V. mimicus* (Spira and Fedorka-Cray 1984). The purified toxin was biologically, immunologically and physicochemically identical to CT except that it appeared to be secreted with an unnicked A subunit. A number of functions have been attributed to these enterotoxins; fluid accumulation in animal models, the ability to elicit diarrhoea in suckling mice and elongation of Chinese hamster ovary cells (De *et al.* 1960; Finkelstein *et al.* 1964; Guerrant *et al.* 1974; Baselski *et al.* 1977).

The CT operon is situated in the *V. cholerae* chromosome and may be present

in multiple copies in strains of the classical biotype, but in a single copy in most strains (70%) of the El Tor biotype (Moseley and Falkow 1980; Kaper and Levine 1981). Duplication of the CT genes (*ctxA* and *ctxB*) in the classical biotype and 2 or more *ctx* copies present on tandemly repeated genetic elements (7kb or 9.7kb) in some strains of the El Tor biotype have been reported (Mekalanos 1983).

The first DNA probes used to detect genes encoding CT were cloned fragments encoding the closely related LT of enterotoxigenic *E. coli* (Moseley and Falkow 1980; Kaper *et al.* 1988a). The DNA probes for the A and B subunits of LT only hybridised to *V. cholerae* under low stringency conditions. These probes gave the first indication that more than one copy of the CT gene existed in some strains of *V. cholerae*, a phenomenon confirmed by Mekalanos (Mekalanos 1983). An LT-A probe was also used to show that nontoxigenic strains lacked any toxin gene sequences and therefore could not revert and serve as a reservoir of cholera (Kaper *et al.* 1981). In molecular epidemiology studies probes were used to demonstrate that O1 strains from the USA had identical restriction fragment patterns homologous to the LT probe and that this pattern was not seen elsewhere in the world. Thus, the restriction fragment length polymorphisms (RFLP) detected by the toxin gene probe demonstrated that a single endemic strain has persisted in the US since at least 1973 and is responsible for sporadic cases of cholera (Kaper *et al.* 1982). With the cloning and sequencing of genes encoding cholera toxin (*ctx*) (Lockman and Kaper 1983; Mekalanos 1983; Lockman *et al.* 1984) the LT probe has been replaced by the CT probe for screening *V. cholerae*. The sequence data showed that LT and CT shared approximately 76% homology. A convenient CT probe was found in the plasmid pCVD27 which contained a 554 bp *Xba*I-*Cla*I fragment encoding 94% of the A subunit gene, ligated to *Eco*RI linkers and cloned into pBR325. CT probes have confirmed and extended observations made with the LT gene probe. Cook *et al.* (Cook *et al.* 1984) using a CTA probe and *Hin*dIII digests showed the classical biotype possessed 2 fragments, the El Tor biotype from eastern countries 1 fragment and the El Tor

biotype from the USA 2 fragments. Similarly Australian *V. cholerae* O1 were found to be genetically diverse and unrelated to strains from Singapore, Jakarta and Louisiana (Desmarchelier and Senn 1989). A large survey of Louisiana isolates yielded a number of *V. cholerae* non-O1 and *V. mimicus* which hybridised with the CT probe. The RFLP in these strains differed from that seen with the Gulf Coast O1 and showed greater genetic diversity than the highly conserved O1 strains (Kaper *et al.* 1986). Based on the toxin gene RFLP and total chromosomal restriction patterns at least two separate clusters of *V. cholerae* non-O1 could be distinguished in addition to a distinct cluster of *V. mimicus* strains.

Strains have been attenuated for use in vaccine development by constructing specific deletions in the genes encoding CT. The cloned CT genes were mutated *in vitro* by removing a 554 bp *Xba*I-*Cla*I fragment containing sequences encoding the A subunit. The promoter for the *ctx* operon and the B subunit sequence were retained, thus allowing the production of the nontoxic B subunit without the A subunit. This mutation was then introduced into the chromosome of virulent *V. cholerae* strains by homologous recombination, specifically replacing the active toxin genes without affecting the other genes encoding critical antigens. The desired mutation was detected using the 554 bp probe to screen for the absence of this sequence. Antibodies directed against the B subunit will prevent binding of CT to intestinal cells. Vaccine candidates derived by this method have proven to be highly immunogenic in volunteers and have conferred excellent protection against challenge with virulent *V. cholerae* (Kaper *et al.* 1984; Kaper *et al.* 1988b). Miller and Mekalanos (Miller and Mekalanos 1984) used a probe for the *toxR* sequence which encodes a positive regulator of CT and possibly other products of *V. cholerae*. When both nontoxigenic and toxigenic strains were probed a 3.9 kb *Eco*RI fragment hybridised to *toxR* sequences in all strains, suggesting that an intact regulatory system for *ctx* genes is present in naturally occurring nontoxigenic strains of both *V. cholerae* O1 biotypes.

Although CT is the main virulence factor in cholera, other extracellular products

may also be involved in the disease process. Recent evidence suggests that CT is part of a "cassette" of virulence factors, including at least two other toxins, Zonula Occludens Toxin (Zot) and Accessory cholera toxin (Ace) (Fasano *et al.* 1991; Baudry *et al.* 1992; Truckis *et al.* 1993).

1.6.2 Toxins associated with CT

CT genes (*ctx*) are inherited as part of a chromosomal "virulence cassette" along with other factors, including Ace and Zot (Fasano *et al.* 1991; Baudry *et al.* 1992; Truckis *et al.* 1993).

Zot is a toxin which increases the permeability of the small intestinal mucosa by affecting the structure of the intracellular tight junction, or zonula occludens; the mechanism is not known. Zot was first discovered in a strain developed for use as a vaccine, CVD101, a CT negative strain of *V. cholerae*, which still caused symptoms including abdominal cramps, vomiting and diarrhoea (Fasano *et al.* 1991). Zot may act by allowing uptake of bacterial endotoxin or other products due to the increased permeability of the tight junctions. The gene encoding Zot was cloned and found to be located immediately upstream of the *ctx* locus (Baudry *et al.* 1992). The *zot* gene encodes a polypeptide of approximately 44 kda. The predicted amino acid sequence shows no homology to any other bacterial toxin, however homology was found with the gene I product of a filamentous bacteriophage believed to be an ATPase (Koonin 1992); the significance of this is not known. The *zot* gene has been found in both *V. cholerae* O1 and non-O1, and does not appear to occur independently from *ctx* sequences (Johnson *et al.* 1993; Karasawa *et al.* 1993).

Another potential enterotoxin is Ace (Truckis *et al.* 1993). The gene for this toxin, *ace*, is located on a 4.5 kb core region which also encodes CT and Zot. The *ace* gene potentially encodes a 11 kda peptide with a predicted amino acid sequence which has homology with a family of eukaryotic ion-transporting ATP-ases, and sequence similarity with a virulence protein of *Salmonella dublin*, SpvB (Krause *et al.* 1991). Ace may act by aggregating and

inserting into the eukaryotic membrane to form an ion channel (Kaper *et al.* 1994).

The genes encoding *ace, zot* and *ctx* are located in tandem on the 4.5 kb core region which is flanked by repetitive sequences (RS1 elements). This arrangement can lead to amplification or deletion of all three toxins as a unit (Goldberg and Mekalanos 1986). This region was shown also to contain a gene encoding an intestinal colonisation factor *cep* (core encoded pilin) (Pearson *et al.* 1993). Interestingly CVD110, a *V. cholerae* O1 El Tor, in which the sequences for Zot, Ace, haemolysin-cytolysin as well as the CT A subunit are deleted, still caused mild to moderate diarrhoea with abdominal cramps and fever when fed to volunteers (Tacket *et al.* 1993). This indicates that the organism possesses other factors that give it diarrhoeagenic potential.

1.6.3 Haemolysin(s) and Thermostable direct haemolysin (TDH)

Haemolysins or cytolysins are thermolabile proteins commonly produced by a number of vibrio species. These extracellular enzymes possess lytic activity against eukaryotic cell lines including erythrocytes and are commonly referred to as haemolysins. *V. cholerae* O1 biotype El Tor was originally distinguished from the classical biotype by haemolysis of sheep erythrocytes (Furniss *et al.* 1978). Strains of *V. cholerae* non-O1 produce a haemolysin which appears to be indistinguishable from haemolysin produced by strains of *V. cholerae* El Tor (Brown and Manning 1985; Yamamoto *et al.* 1986; Ichinose *et al.* 1987). Also, strains of *V. mimicus* produce a haemolysin which has partial identity with these haemolysins (Shinoda *et al.* 1993; Uchimura *et al.* 1993). Purified haemolysin produced by strains of *V. cholerae* El Tor is lethal for mice, causes increased vascular permeability in rabbits and lyses erythrocytes (Honda and Finkelstein 1979). The haemolysin causes fluid accumulation in ligated rabbit ileal loops and, in contrast to the watery fluid produced in response to CT, the fluid is bloody with mucus (Ichinose *et al.* 1987).

A cytolysin that is cytotoxic for Y1 adrenal and CHO cells, and causes fluid

accumulation in rabbit ileal loops has been described by McCardell *et al.* and Spira *et al.* (McCardell *et al.* 1985; Spira *et al.* 1986). This cytolysin was thought to be identical to El Tor haemolysin. However this cytolysin has recently been shown to form anion-selective channels in planar lipid bilayers (Krasilnikov *et al.* 1992).

V. cholerae and *V. mimicus* probably produce more than one haemolysin or cytolysin. Haemolysins are possible virulence factors, particularly for *V. cholerae* non-O1 and *V. mimicus* that do not produce CT. The *hlyA* gene encoding haemolysin, is found in both biotypes of *V. cholerae* O1 and in *V. cholerae* non-O1 (Brown and Manning 1985). The *hlyA* gene has been cloned by several groups (Goldberg and Murphy 1984; Manning *et al.* 1984) and its DNA sequence has been determined. DNA sequence analysis of the *hlyA* gene predicts a precursor protein of 82 kda which is processed by proteolysis to a final active form of 65 kda (Hall and Drasar 1990). Using a 6.2 kb *Pst*I probe with Southern blots, Brown & Manning (Brown and Manning 1985) have shown homologous sequences in chromosomal restriction digests of *V. cholerae* non-O1 strains. Both haemolytic and non-haemolytic strains possess sequences homologous to the probe suggesting mutations in a regulatory rather than structural gene. Differences in sizes of restriction fragments hybridising with the haemolysin probe can distinguish haemolytic from non-haemolytic strains of both O1 and non-O1 *V. cholerae*. However the importance of haemolysin in diarrhoea remains uncertain; in volunteer studies, ingestion of a *V. cholerae* O1 vaccine strain not producing CT and mutated in the *hlyA* gene by deletion of a 400 bp *Hpa*1 fragment, still caused diarrhoea in 33% of the subjects (Levine *et al.* 1988).

Another well-characterised haemolysin known as the Thermostable Direct Haemolysin (TDH), is associated with the pathogenesis of *Vibrio parahaemolyticus*. It can be detected by the Kanagawa phenomenon in which lysis is shown on Wagatsuma agar containing human blood. *V. cholerae* non-O1 produce a TDH which has been designated NAG-rTDH and resembles the TDH of *V. parahaemolyticus* (Vp-TDH) (Honda *et al.* 1985; Yoh *et al.* 1985;

Yoh *et al.* 1986). TDH was purified using immunoaffinity column chromatography and anti-(Vp-TDH)-antibody (Honda *et al.* 1985). The electrophoretic mobilities of Vp-TDH and NAG-rTDH haemolysin are similar on SDS-slab electrophoresis. Their lytic activities on erythrocytes of various animals are also similar; rabbit, chicken and human erythrocytes are very sensitive whereas horse erythrocytes are completely resistant. Vp-TDH and NAG-rTDH are also closely related immunologically.

NAG-rTDH genes are carried on a large plasmid in *V. cholerae* non-O1 (Honda *et al.* 1986). This is of special interest as Vp-TDH is chromosomal (Nishibuchi *et al.* 1985). Transmission of the *tdh* gene could explain the presence of TDH-related haemolysins in *V. cholerae* non-O1, *V. mimicus* and *V. hollisae* (Yoh *et al.* 1986; Nishibuchi *et al.* 1990; Terai *et al.* 1990).

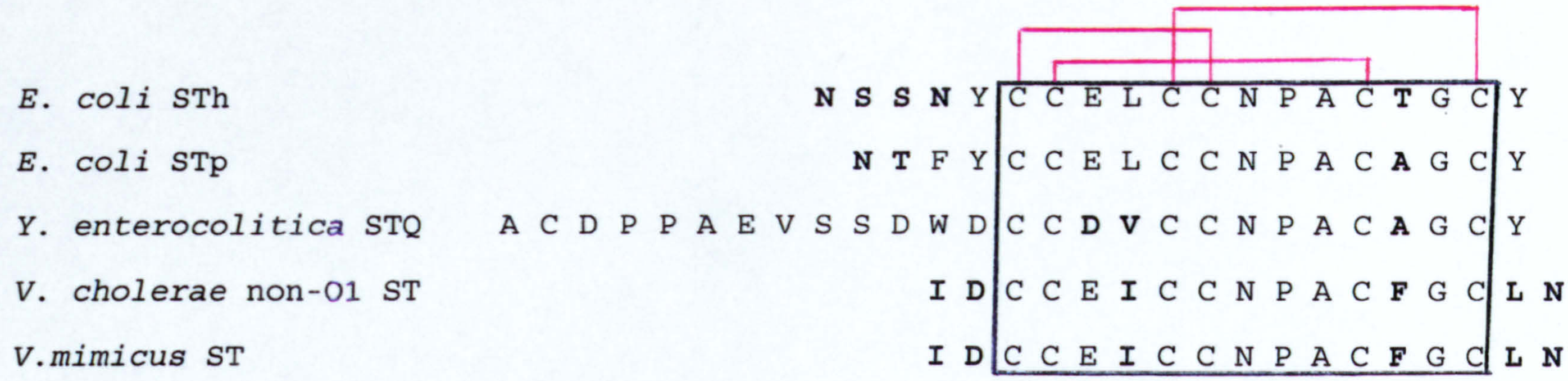
1.6.4 Heat-stable enterotoxin (ST)

Spira and Daniel (Spira and Daniel 1979) and Nishibuchi *et al.* (Nishibuchi *et al.* 1983) both reported the production of a toxin with activity in the infant mouse assay. Spira and Daniel found *V. cholerae* non-O1 strains which produced ST similar to that of *E. coli* with a peak fluid accumulation (FA) by 4 hours in the suckling mouse assay and the rabbit ileal loop assay. However, the factor responsible for FA in the study of Nishibuchi *et al.* was only detected when whole cell cultures were administered and not in filtrates of bacterial cultures. The FA in the latter study could have been caused by a number of virulence factors but probably not an ST-like toxin. ST comprise an expanding family of structurally, functionally and immunologically related peptide toxins (Figure 2) which bind specifically and reversibly to a receptor found in the microvillus membranes of the intestinal cell brush border stimulating fluid secretion via receptor-mediated activation of guanylate cyclase (Field 1978).

The presence of a heat-stable enterotoxin has been demonstrated in *V. cholerae* non-O1 (Honda *et al.* 1985; Arita *et al.* 1986) and in *V. mimicus* (Arita *et al.* 1991). These two

heat-stable toxins were designated NAG-ST and VM-ST respectively. Both NAG-ST and VM-ST were indistinguishable on HPLC column chromatography suggesting their close similarity. NAG-ST is a 17 amino acid protein which is immunologically related to the ST of enterotoxigenic *E. coli* (ETEC) (Honda *et al.* 1985). ETEC ST causes diarrhoea which may be severe (Sack 1975) and it is possible that NAG-ST plays a part in the severe watery diarrhoea induced by some *V. cholerae* non-O1. Amino acid sequences of purified NAG-ST and VM-ST toxin were found to be similar to that of *E. coli* ST which is a known virulence factor. From position 3 to 15 (shown as the boxed area in Figure 2) the sequence is almost identical, suggesting that this sequence is essential for expression of enterotoxic activity (Takeda *et al.* 1979; Takao *et al.* 1983; Yoshimura *et al.* 1986). The traditional method for detecting STI type toxins (STh and STp) in ETEC has been the infant mouse assay (Dean *et al.* 1972). Using this method Arita *et al.* found 55% (55/100) human and 62% (5/8) of *V. cholerae* non-O1 from the environmental sources in Southeast Asia produced NAG-ST (Arita *et al.* 1986). However, by hybridisation with a mixed oligonucleotide probe, Hoge *et al.* found only 6.8% (7/103) of the *V. cholerae* non-O1 isolates from Thailand, and none of the 78 isolates from Mexico and USA, were positive for NAG-ST (Hoge *et al.* 1990). The nucleotide sequence for this mixed oligonucleotide probe was deduced from the amino acid sequences of *E. coli* STh and STp. Although the amino acid sequences of the ST family are similar (Takao *et al.* 1985) the nucleotide sequences of the genes encoding them are distinctly different, with only 50% homology (Ogawa *et al.* 1990). This may explain the failure to detect NAG-ST using *E. coli* ST probes (Seriwatana *et al.* 1987; Sommerfelt *et al.* 1988). In 1990 the NAG-ST gene was cloned and sequenced enabling the construction of a 271 bp cloned fragment of the NAG-ST gene (Ogawa *et al.* 1990). Recently Pal *et al.* used this cloned DNA probe to assess the prevalence of NAG-ST among environmental strains isolated in Calcutta (Pal *et al.* 1992). They found 2.3% (12/521) of strains hybridised and suggested that there might be a correlation between serogroup and NAG-ST. Although it is exceedingly uncommon to find ST

Figure 2: Heat-stable toxin amino acid sequences



Amino acid sequence differences are highlighted. The activity of ST can be attributed to a stretch of sequences composed of the 13 amino acid residues (the boxed area). Disulphide bonds are shown in red.

in *V. cholerae* O1 one study did identify this toxin in 1 of 197 isolates of *V. cholerae* O1 (Takeda *et al.* 1991). The gene encoding the ST of O1 was cloned and found to be similar to the NAG-ST gene (Takeda *et al.* 1991). The ST of *V. mimicus* and the Hakata group of *V. cholerae* non-O1 (now designated serogroup O140) were analysed with respect to their amino acid sequences. Vm-ST was identical to NAG-ST (Arita *et al.* 1991) whereas Hakata-ST was one amino acid longer (Dohi *et al.* 1993).

Although, studies using gene probes for ST have only found a low incidence of the ST gene in *V. cholerae* (Hoge *et al.* 1990; Pal *et al.* 1992), strains which produce this toxin have the potential to produce severe diarrhoea. It was demonstrated that a *V. cholerae* non-O1 strain which produced NAG-ST caused severe diarrhoea in a volunteer study (Morris *et al.* 1981).

1.6.5 Cytotoxins

Cytotoxins are biologically and physiologically distinct from cytolysins. In contrast to cytolysins, cytotoxins have been described in only a limited number of vibrio species, including *V. cholerae* and *V. fluvialis*, and are characterised principally by their ability to cause cell death without lysis (Janda *et al.* 1988). Most *V. cholerae* non-O1 strains produce cytotoxin(s), including the CT producing strains (Said *et al.* 1994). When cytotoxic activity is produced it will often mask the rounding effects of cytotonic toxins like CT. The *V. fluvialis* cytotoxin is a heat-labile substance which causes elongation of CHO cells (Lockwood *et al.* 1982; Wall 1984).

A specific cytotoxin related to Shiga-like toxin or Vero cytotoxin (VT)-like toxin appeared to be demonstrated in cell lysates of *V. cholerae* and *V. parahaemolyticus*, using cytotoxicity tests and neutralisation assays with anti Shiga-toxin antiserum (O'Brien *et al.* 1984). It is possible that this toxin may play a role in bloody or dysentery-like diarrhoea occasionally associated with *V. cholerae* non-O1.

1.6.6 Colonisation factors

Colonisation of the epithelial mucosa of the small intestine appears to be a critical step in the ability of organisms, such as *V. cholerae* O1 and *V. cholerae* non-O1, to elicit diarrhoea (Spira and Fedorka-Cray 1983). A number of possible mechanisms involved in the penetration of the mucous layer and the colonisation of the epithelial cells have been proposed, these include; chemotaxis, motility through a single polar flagellum, haemagglutinins, outer membrane proteins, lipopolysaccharides and pili or fimbriae. However, investigations into cell-associated factors have been complicated by the difficulty of linking the presence of such factors in infecting strains, with overt pathogenesis. Virulence factors may have multiple biologic functions or properties, thereby making the study of such factors even more difficult.

The mucous layer which protects the small intestine is chemotactic to vibrios (Freter and Jones 1976; Freter 1981) and these organisms are known to produce proteolytic enzymes including mucinase (Booth and Finkelstein 1986). These factors may be important in allowing vibrios to traverse the mucous layer.

It has also been hypothesised that the flagellum plays a key role in adhesion by aiding both, penetration of the mucous barrier and colonisation of the epithelial surface (Freter 1981; Attridge and Rowley 1983). Early studies suggested that non-motile variants of *V. cholerae* O1 were less virulent. The flagellum may function merely as an agent of motility, serving to promote interaction between cell-surface adhesins of vibrios and the intestinal surface (Guentzel 1975). Alternatively this structure may bear the adhesins and thus be more directly involved in the actual binding event (Jones and Freter 1976). The study of Jones & Freter demonstrated that non-motile variants derived from motile strains of *V. cholerae* lacked the capacity of the parental strain for binding to isolated brush-border membranes or ileal slices *in vitro* (Jones and Freter 1976). Using antisera of different specificities Attridge & Rowley demonstrated independently both binding and motility functions associated with the flagellar

structure (Attridge and Rowley 1983). The observation of immobilized yet adherent vibrios, or alternatively motile nonadherent ones, showed that motility was neither necessary nor sufficient for *in vitro* attachment. Other studies have failed to corroborate the findings that non-motile strains were less virulent (Teppema *et al.* 1987). On scanning electron micrographs flagella are clearly protruding into the luminal space and not involved in attachment (Sack 1992).

The most recent research suggests that pili are the critical factors in colonisation. Several groups have reported the presence of pili in *V. cholerae* O1 (Ehara *et al.* 1987; Taylor *et al.* 1987; Hall *et al.* 1988; Pearson *et al.* 1993), *V. cholerae* non-O1 (Honda *et al.* 1988) and *V. mimicus* (Uchimura and Yamamoto 1992). These filamentous surface organelles mediate the specific binding and colonisation of host tissue and are essential components in the virulence of many pathogenic bacteria (Beachey 1981). The best characterised pilus of *V. cholerae* is the toxin co-regulated pilus (TCP) (Taylor *et al.* 1987; Attridge and Voss 1993). Under the electron microscope each pilus is 5-7 nm in width and 10-15 μm in length and due to their hydrophobic nature they aggregate to form large bundles. The expression of TCP bundles causes autoagglutination and sedimentation in broth culture as well as conferring the ability to haemagglutinate erythrocytes from CD-1 mice in the presence of fucose or mannose. These sugars inhibit the majority of other *V. cholerae* haemagglutinins. As the name implies TCP is co-regulated with CT. At the molecular level the synthesis of the 20.5 kda protein pilin subunit (designated TcpA) is coordinately expressed within a virulence regulon by the transmembrane DNA-binding regulatory protein ToxR and by a recently identified global regulator ToxT. The role of TCP in colonisation has been established for both *V. cholerae* O1 biotypes in volunteer studies. Mutant strains which did not produce pili failed to colonise the intestine and failed to produce an effective immune response (Herrington *et al.* 1988). Immunoblotting has shown that TcpA is expressed *in vivo* (Kauffman and Taylor 1994). The receptors for TCP have not been identified. TcpA shows homology to type IV pilins, which are produced by a number of bacterial pathogens including *E. coli* (Giron *et al.* 1991),

Pseudomonas aeruginosa (Sastry *et al.* 1983) and *Neisseria gonorrhoea* (Meyer *et al.* 1984). Type IV pili are thought to be critical for virulence in *E. coli*, *P. aeruginosa* and *N. gonorrhoea*.

Another pilus which is closely associated with CT is the recently identified core-encoded pilus (*cep*) which is found alongside *ctx*, *ace* and *zot* (Pearson *et al.* 1993). *V. cholerae* O1 also produce other pili which have haemagglutinating activity, but no apparent role in colonisation (Iwanaga *et al.* 1989). Likewise *V. cholerae* non-O1 have been shown to produce pili which are closely related to the TCP pili but which have no role in colonisation (Nakasone and Iwanaga 1990).

The role of other attachment structures such as adhesins (Faris *et al.* 1982; Kabir and Ali 1983), some of which mediate the agglutination of erythrocytes, is less clear since they are found in a broad range of serogroups of diverse origin (Booth and Finkelstein 1986) and fail to show a distinct association with the infectious process (Attridge and Rowley 1983).

A protease, which may also play a role in dissolving the intestinal mucous gel (Finkelstein and Hanne 1983), and cell-associated soluble haemagglutinins (Hanne and Finkelstein 1982) have the potential to be involved in the adherence or colonisation of *V. cholerae*. A protease similar to that of *V. cholerae* O1 has been described in *V. cholerae* non-O1 (Booth and Finkelstein 1986; Honda *et al.* 1987). Booth & Finkelstein (Booth and Finkelstein 1986) examined the distribution of a variety of haemagglutinins as potential colonisation factors in strains of *V. cholerae* O1 and non-O1. *V. cholerae* O1 secrete a major protease which is also a haemagglutinin (HA/protease). HA/protease has been characterised as a zinc-dependent metalloprotease (Booth *et al.* 1983) with the ability to nick (activate) the A subunit of CT (Booth *et al.* 1984) and to cleave or digest other putative physiologically important substrates, including mucin, fibronectin and lactoferrin (Finkelstein *et al.* 1983). Protease deficient mutants are known to be less virulent (Schneider and Parker 1978).

Additionally it has been suggested that cell-associated mannose-sensitive, fucose-sensitive, and mannose-fucose-resistant haemagglutinins may be virulence factors in pathogenic *V. cholerae* (Jones and Freter 1976; Bhattacharjee and Srivastava 1978; Hanne and Finkelstein 1982; Attridge and Rowley 1983). Booth & Finkelstein concluded that although the HA/protease and cell-associated haemagglutinins were widely distributed in *V. cholerae* O1 and non-O1, these factors are not sufficient for virulence. Interestingly HA/protease has been shown to be related to the elastase of *Pseudomonas aeruginosa* (Hase and Finkelstein 1990). Elastase also has both proteolytic and haemagglutinating activity, and appears to be involved in tissue destruction in Pseudomonas infection. Cholera is not an invasive process, and there is no evidence that tissue damage is involved. The HA/protease may serve functions other than tissue damage, such as participation in adherence and detachment in *V. cholerae* O1 infections. Although the organisms which cause cholera are not invasive, other pathogenic vibrios involved with extraintestinal disease with a potential for invasiveness (Booth and Finkelstein 1986) may use HA/protease in a destructive way.

A neuraminidase (NANase) found in *V. cholerae* O1 may have a role both in adhesion and in binding and penetration of CT into intestinal epithelial cells (Holmgren 1975). NANase catalyses the conversion of higher order gangliosides to toxin receptor GM1 (Holmgren 1975). Galen *et al.* demonstrated that deletion of NANase does not decrease the biological activity of CT (Galen *et al.* 1992). The production of CT and expression of TCP are regulated by diverse environmental signals (Miller and Mekalanos 1984; Waldor and Mekalanos 1994), it appears that when environmental conditions do not promote optimal expression of CT or TCP the ability of NANase to enhance binding of CT could contribute to the pathogenic process (Galen *et al.* 1992). *V. cholerae* O1 biotype El Tor produce higher ratios of mild or asymptomatic infections than classical strains (Gangarosa and Moseley 1974). The El Tor biotype also produces less NANase with lower specific activity than that produced by classical strains (Kabir 1984). Thus NANase may be an accessory

virulence factor of *V. cholerae* which enhances pathogenicity when the influence of primary factors is reduced.

A recent report (Panigrahi *et al.* 1990) suggested that pathogenic *V. cholerae* non-O1 could be distinguished from non-pathogenic strains by adhesion on Caco2 cells. This is a human enterocyte-like cell line derived from a moderately differentiated human colonic adenocarcinoma. It has proved a useful *in vitro* model for adhesion of pathogens such as *Listeria monocytogenes* and *E. coli*. Although the mechanisms of colonisation of *V. cholerae* are not fully characterised, it may be that, similar to diarrhoeagenic *E. coli*, more than one mechanism of adherence will be found.

1.6.7 Lipopolysaccharide (LPS) and Outer membrane proteins (OMP)

The ability of strains of *V. cholerae* to express long-chain lipopolysaccharide is only poorly understood, although strains belonging to serogroups O1 and O139 are thought to express LPS. For these organisms, the ability to produce LPS may be considered a virulence factor since only strains of *V. cholerae* belonging to serogroups O1 and O139 cause epidemic cholera. Purified *V. cholerae* O1 LPS has been found to cause haemagglutination, which can be inhibited by monoclonal antibodies directed against LPS (Booth *et al.* 1986). Although the role of LPS as an adhesive factor has not been established, antibodies produced against *V. cholerae* LPS are protective in animal models, and the observed protection may involve interference with LPS during mucosal colonisation (Booth and Finkelstein 1986).

The somatic antigen or LPS may be a useful indicator of diarrhoeagenic potential within the *V. cholerae* non-O1. In 1980 the WHO (WHO Scientific working group 1980) reported a prevalence of the O5 serogroup in gastrointestinal disease and O8 in the environment, while Donovan (Donovan 1984) found O2, O5, O7 and O37 predominated in gastrointestinal disease and O4 predominated in the environment. It remains to be determined whether certain serogroups are inherently more pathogenic than others, and whether certain

serogroups are more prevalent in certain geographic areas. The frequency of certain serogroups as a cause of gastroenteritis may indicate a more virulent group or simply reflect their common occurrence in environmental samples.

The outer membranes of vibrios, like most gram-negative bacteria, contain several major proteins (OMP) some of which are strongly antigenic (Kabir 1980; Sengupta *et al.* 1989). Kabir reported a major protein which migrated on SDS-PAGE gels as a band of 48 kda, which was common to all strains of *V. cholerae* O1. Kelley & Parker found a 45 kda major protein which appeared to be the major structural protein of the outer membrane (Kelley and Parker 1981). Using SDS-PAGE, strains of *V. cholerae* have a distinct OMP pattern when compared to many other gram-negative bacteria (Manning *et al.* 1982). SDS-PAGE profiles of OMs produced from strains of *V. cholerae* O1 are generally comprised of OMPs in the range 25 - 45 kda range, and also additional minor proteins. *V. cholerae* non-O1 are considerably more heterogeneous. Nevertheless, a protein which migrates during SDS-PAGE as a band of 25 kda appears to be common to all strains of *Vibrio* (Manning and Hayes 1984).

Some studies have suggested that the flagellar sheath is a continuation of the outer membrane lipid bilayer in that it contains LPS (Fuerst and Perry 1988) and specific proteins (Hranitsky *et al.* 1980). Although the significance of most of these OMPs in virulence is not known, a role in colonisation has been suggested for an iron-regulated OMP (Goldberg *et al.* 1990). It has also been reported that OMPs are regulated by the ToxR gene which regulates a variety of virulence factors including CT and TcpA (Taylor *et al.* 1987).

1.6.8 High affinity iron uptake.

Iron is an essential element for most aerobic organisms. Pathogenic bacteria must be able to obtain iron from the host for bacterial multiplication to occur. Iron in the ferric form is almost completely insoluble in aqueous solutions, and specialised transportation and storage mechanisms have evolved. In the mammalian host, iron forms an integral part of haem

molecules in erythrocytes. In addition, iron is also stored intracellularly in the liver, as hemosiderin and ferritin; whilst in blood and mucus iron is transported by specialised iron-binding glycoproteins, transferrin and lactoferrin. To obtain sufficient iron from the host, pathogenic bacteria must either be able to compete for iron bound to transferrins, and/or obtain iron from iron-containing compounds such as haemoglobin. Haemolytic toxins or haemolysins may make iron available to an invading pathogen by releasing intracellular iron compounds such as haemoglobin, heme or ferritin. The synthesis of some toxins has been shown to be regulated by iron, and maximal expression occurs at low iron concentrations.

Most pathogenic bacteria possess at least one high affinity iron uptake system, which involves the production of low molecular weight, high affinity iron chelating molecules (termed siderophores) and inducible outer membrane protein receptors for binding ferric-siderophores (Lankford 1973; Neilands 1981). Siderophores have an iron binding affinity considerably higher than host iron transport glycoproteins and can effectively remove iron from the host iron stores for transport into the bacterial cell. These low molecular weight compounds have extremely high affinities for iron and are synthesised in response to iron limitation. The two major siderophores are the phenolic compound enterobactin and the hydroxamate siderophore aerobactin. The synthesis, transport and regulation of expression of two of these compounds, enterobactin and aerobactin, and their role in pathogenicity has been studied in detail in *E. coli*. The siderophore enterobactin, formed from chorismic acid, has the highest iron binding affinity known; however, in removing iron from ferric-enterobactin the molecule becomes destroyed as an active iron carrying ligand. In contrast, aerobactin has a much lower affinity for iron than enterobactin, but has the advantage of being reusable. Indeed, most invasive bacteria have been shown to express an aerobactin-mediated iron uptake system, suggesting that possessing a recyclable iron uptake system may be advantageous. Although iron transport systems are less extensively characterised in other bacterial species, there appears to be considerable variety in the mechanisms by which pathogenic bacteria acquire essential iron.

In vibrios iron acquisition systems have been investigated in *V. anguillarum*, *V. vulnificus* and *V. parahaemolyticus* (Crosa 1980; Andrus *et al.* 1983; Tomalsky and Crosa 1984; Chart and Griffiths 1985; Morris *et al.* 1987). The role of iron transport has been most clearly defined for *V. anguillarum* and less clearly defined for other vibrio species. *V. cholerae* produces a phenolic siderophore, vibriobactin (Payne and Finkelstein 1978; Griffiths *et al.* 1984) that is unusual in that it contains norspermidine, a polyamine rarely found in bacteria (Griffiths *et al.* 1984). Biosynthesis of the vibriobactin moiety shares similarities with enterobactin synthesis. Mutants of *V. cholerae* blocked in chorismate synthesis fail to produce vibriobactin (Sigel *et al.* 1985), and the cloned vibriobactin genes complement *E. coli entA,C* mutations. In addition to the siderophore, at least five outer membrane proteins (Sigel and Payne 1982) and haemolysin (Stoebner and Payne 1988) are maximally expressed under iron-limiting conditions. *V. cholerae* isolated from the intestinal lumen of infected rabbits express outer membrane proteins that are induced *in vitro* under iron-restricted conditions indicating that iron limitation occurs *in vivo* (Sciortino and Finkelstein 1983). However, synthesis and transport of vibriobactin are not essential for *in vivo* multiplication or virulence. When tested *in vivo* in the infant mouse model, mutants defective in vibriobactin synthesis or transport retained the ability to multiply and cause diarrhoea (Sigel *et al.* 1985). *V. cholerae* is a noninvasive surface pathogen and may be able to acquire sufficient iron in the intestine in the absence of siderophore synthesis. *V. cholerae* was found to use haemin or haemoglobin as a sole source of iron by a siderophore-independent mechanism and may acquire iron from these compounds *in vivo* (Stoebner and Payne 1988).

1.6.9 Extrachromosomal elements

The genetic basis for the production of many of the putative virulence factors of *V. cholerae* non-O1 is not known. However, all the virulence factors so far examined in *V. cholerae* O1 are encoded on a chromosome of approximately 2.8 x 10^3 kb and the gene

structure is similar to that found in other enteric bacteria (Kaper and Baldini 1992).

Plasmid carriage is infrequent among Vibrionaceae. Most are of low molecular weight and are cryptic (Janda *et al.* 1988). High molecular weight, multiple-antibiotic resistance plasmids are occasionally found in *V. cholerae*. *V. cholerae* can stably maintain plasmids belonging to the incompatability group IncC and rarely IncJ (Hedges *et al.* 1977). Multiple drug resistance, encoded by conjugative plasmids belonging to IncC has been reported in strains from Bangladesh (Glass *et al.* 1983b). The first outbreak of multi-resistant *V. cholerae* O1 El Tor occurred in Tanzania in 1977. Subsequently there have been outbreaks in Bangladesh. In both instances the resistance was encoded by a 100 Mda conjugative plasmid belonging to the Inc C group (Threlfall *et al.* 1980; Threlfall and Rowe 1982). The emergence of multiresistant strains of *V. cholerae* O1 has also been reported from Ecuador (Threlfall *et al.* 1993). Attempts to correlate extrachromosomal elements with phenotypic or virulence characteristics have been unsuccessful (Newland *et al.* 1984). Hamood *et al.* (Hamood *et al.* 1986) demonstrated that the R factor pVH2 (a large conjugative plasmid of compatibility group C (Threlfall *et al.* 1980)) of *V. cholerae* enhanced virulence in infant mice but this effect was not related to CT, haemolysin or colonisation factors. Conversely the sex factor P of *V. cholerae* causes a decrease in pathogenicity by reducing CT production (Newland *et al.* 1984; Guidolin and Manning 1987). The only toxin shown to be extra-chromosomal in *V. cholerae* non-O1 is the thermostable direct haemolysin (NAG-rTDH) (Honda *et al.* 1986). The gene, *tdh*, was shown to exist on a large, 33 kb, plasmid by hybridisation with a *tdh* gene probe derived from an internal region of the *V. parahaemolyticus tdh* structural gene (Nishibuchi *et al.* 1985; Nishibuchi and Kaper 1985). The *tdh* gene present on a large plasmid in *V. cholerae* may have originated from the chromosome of *V. parahaemolyticus*. The transmission of *tdh* may be plasmid mediated and take place in the aquatic environment. This transfer of genetic material may be infrequent as the incidence of NAG-rTDH among *V. cholerae* non-O1 clinical isolates is quite low (Yoh *et al.* 1985). The molecular basis for the exclusion and instability of most plasmids in *V. cholerae* is not known, and the apparent lack of plasmids may be related to

the practical difficulty in isolating extrachromosomal material from vibrios.

The cross-resistance of trimethoprim and O/129 of strains of *V. cholerae* is associated with the acquisition of a trimethoprim resistant dihydrofolate reductase. Transposable elements have been isolated from *V. cholerae* O1; the resistance of an El Tor strain to trimethoprim and the vibriostatic agent O/129 (pteridine compound 2,4 - diamino - 6,7 -diisopropyl pteridine) is due to a transposon inserted into the chromosome (Goldstein *et al.* 1986). An O/129 resistant *V. cholerae* non-O1 has been isolated from a patient with gastroenteritis, this strain was also resistant to trimethoprim (Abbott *et al.* 1992).

A 2.7 kb element designated RS1 (repetitive sequence 1) is found flanking the gene for CT. Transposition of this element has been observed and this element appears to be involved in the amplification of *ctx* (Mekalanos 1983; Betley 1986).

1.7 Scope of this study

Little is known about the virulence determinants of strains of *V. cholerae* non-O1 and the closely related *V. mimicus*. The ability of these organisms to produce CT is clearly involved in the pathogenesis of cholera and cholera-like diseases; however, the pathogenic mechanisms of *V. cholerae* non-O1 and *V. mimicus* have not been fully elucidated. During the last decade, however, it has been recognised that other factors may be involved in the virulence of these organisms. Although epidemic strains (O1 and O139) are closely associated with CT, some outbreaks and many sporadic infections are caused by "non-toxigenic" serogroups, other than O1 or O139. A number of toxins, other than CT, and a number of possible adhesive factors have been postulated as having a role in the disease process.

The aim of this investigation was to identify virulence properties associated with *V. cholerae* non-O1 and *V. mimicus*, and to establish a correlation, if any, with serogroup. Strains were characterised by biotype and serotype. Appropriate strains were surveyed to detect toxins and other known virulence factors. The work included; development of techniques and identification of new virulence factors.

MATERIALS AND METHODS

2.1 Bacterial strains

Strains of *V. cholerae* non-O1 and *V. mimicus*, from human and environmental sources, were from the Laboratory of Enteric Pathogens (LEP) culture collection (Appendix 2 - Strain catalogue). Strains of *V. cholerae* O1 were also from the LEP culture collection.

A panel of reference strains of *V. cholerae* belonging to serogroups O2 - O139, and a rough strain (CA385) were kindly provided by Dr. R. Sakazaki and Dr. T. Shimada (Sakazaki and Donovan 1984; Shimada *et al.* 1994). The strains are listed in Table 2a and 2b.

V. cholerae strain WBDV-101E (serogroup O49, CT+), was kindly provided by Dr. P. Echeverria, and used as a CT-positive non-O1 control strain. *V. cholerae* strain E51116 (O1 classical from Bangladesh CT+) from the L.E.P. culture collection, was used as a CT-positive *V. cholerae* O1 control. *Escherichia coli* strain B7A (O148:H28) (Dupont *et al.* 1971) was used as an LT and ST positive control.

Strains of *V. cholerae* belonging to serogroups O6 and O23 and known to produce CT, had been isolated in Australia and were kindly provided by Dr. P. Desmarchelier. Strain NAG 8-2E derived from A5 (Arita *et al.* 1986), kindly provided by Dr. T. Honda, was used as a NAG-ST positive *V. cholerae* non-O1 control strain.

2.1.1 Storage of bacterial strains

All strains of *V. cholerae* were maintained on nutrient agar slopes at room temperature. *E. coli* strain B7A was stored on a Dorset Egg slope at room temperature. Strains which were of particular interest, such as toxin positive strains, were also freeze-dried (lyophilised).

2.1.1a Lyophilisation

Cultures were inoculated onto agar slopes and incubated (37 °C, 16 h). The resultant cell growth suspended in 2 mls Difco nutrient broth containing 0.5% inositol. 0.2 ml of these suspensions was added to freeze-drying tubes (100mm x 7mm) which were plugged with sterile cotton wool prior to a preliminary drying for 4 h using an Edwards model 5PS freeze drying machine. After this the tubes were replugged with sterile cotton wool and the neck of the tubes constricted between the plug and the top and put on a multiple drier for a further 20 h. After the second drying period tubes were sealed at the constriction and stored in the dark. Freeze dried cultures were checked for serogroup and toxin type to ensure that strains had retained their original phenotype.

2.2 Identification and typing of *V.cholerae* and *V.mimicus*

Strains of *V. cholerae* and *V. mimicus* were identified biochemically and characterised with respect to biotype, serotype, phage-type and antibiotic resistance type (R-type).

2.2.1 Biochemistry

Bacterial strains were tested for their ability to utilise mucate and to ferment adonitol, arabinose, cellobiose, dulcitol, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose and xylose. They were also tested for their biochemical reactions in the following tests: ONPG (o-nitrophenyl-ß-D-galactopyranoside) test, citrate utilization on Simmons citrate and Christensens citrate agar slopes, acetate utilization on sodium acetate agar slopes, urease activity, arginine dihydrolase production, lysine and ornithine decarboxylase production, indole production, hydrogen sulphide (H_2S) production in glucose iron agar, growth in the presence of potassium cyanide (KCN), malonate utilization, PPA test (deamination of phenylalanine to phenylpyruvic acid), gluconate

oxidation, aesculin hydrolysis, gelatin hydrolysis, motility, oxidation-fermentation test in two tubes of Hugh & Leifson medium, nitrate reduction, Vogues-Proskauer test (acetylmethylcarbonil production), oxidase activity, and O\129 sensitivity. (See Appendix 1 for further details).

2.2.2 Biotyping

Strains of *V.cholerae* belonging to serogroup O1 are designated as classical and El Tor biotypes on the basis of six minor biochemical characters, as shown in Table 3 (Furniss *et al.* 1978). *V.cholerae* non-O1 and *V.mimicus* were also tested for these characteristics.

2.2.2a Haemolysis

Haemolytic properties were detected using horse red blood cells (rbc), freshly washed in saline and suspended in saline to give a blood cell concentration of 20%. A 1 ml volume of this was added to 20 mls of saline. The suspension was mixed with melted nutrient agar (4%), equilibrated to 60 °C to avoid blood cell denaturation, and poured into sterile petri-dishes and allowed to dry. 10 µl volumes of a 3 h bacterial culture, grown in nutrient broth, were spotted onto the plate alongside haemolysis positive and negative control strains. After incubation at 37 °C overnight plates were examined for lysis. Any haemolysis visible by eye was recorded as positive. The size of the zone of haemolysis in mm was measured and it was noted if there was any "greening", complete clearing or opacity.

Haemolysis of rbc's, other than those from horse, was tested using a microtitre method. Equal volumes (25 µl) of 1% washed rbc and bacterial filtrates were mixed gently and the plate incubated at 37 °C for 4 h. When lysis of cells occurred the well contents retained a red/brown colour; when the well contents became clear with the cells forming a pellet in the base of the well, the strain was considered non-haemolytic.

Table 4: Biotypes of *V.cholerae* O1

Biochemical tests	classical	El Tor
Haemolysis	-	+
VP	-	+
Chick cell agglutination	-	+
Polymyxin	S	R
classical phage 3	S	R
El Tor phage 4	R	S

S = sensitive

R = resistant

2.2.2b Vogues-Proskauer test

Strains were grown in 3 mls alkaline peptone water for 3 h and approximately 100 µl "stabbed" into tubes of Vogues-Proskauer (VP) agar medium using a sterile pasteur pipette, prior to incubation at 30 °C overnight. For the VP test, 0.2 mls of solution A (Appendix 1) and 0.1 mls of solution B (Appendix 1) were added to the tubes and left at room temperature for 15 min. The appearance of a deep red ring at the top of the agar indicated a positive reaction.

2.2.3 Serology

Strains of *V.cholerae* were serotyped, based on the somatic or 'O'-antigens, according to the method of Sakazaki and Donovan (Sakazaki and Donovan 1984). There are 140 known serogroups of *V. cholerae* (Shimada *et al.* 1994); however, for this study sera against only the first 83 serogroups and O139 were available. Antisera were produced to strains of *V. cholerae* belonging to serogroups O1 to O83 and O139, using heat-killed organisms. The O-antigen is thermostable (100 °C for 2 h) but there is a reduction in O-agglutinability after 30 min. Live cultures were not used as these may not be agglutinated by O-antiserum due to the presence of a surface mucoid substance. Strains of *V. cholerae* which did not produce O-antigen, termed "O-rough", give identical serology results. This was thought to be due to cross-reactions with core-LPS antigens. To avoid these cross-reactions, antisera were all absorbed with an O-rough type strain (CA385) prior to use.

Flagellar or 'H' antigens are heat-labile, and incubating bacterial preparations at 100 °C for 2 h enables the detection of O-antigen agglutination reactions in the absence of H-agglutination. Members of the genus *Vibrio* share common H-antigens, therefore anti-flagellar antibodies have little value for the serotyping of strains of *V. cholerae* or *V. mimicus*.

2.2.3a Preparation of antisera

For the preparation of bacteria for producing rabbit antibodies to the somatic antigens, strains of *V. cholerae* were grown on nutrient agar slopes (37 °C, overnight). For each strain, the cell mass was washed from agar slopes into 12 mls of 1% aqueous saline. These bacteria were incubated at 100 °C for 2 h to denature flagellar antigens and the cells sedimented by centrifugation at 2000 *g* for 30 min. The supernatants were discarded and the procedure repeated twice. The cells were then suspended in 25 mls 1% saline. Antisera were prepared in New Zealand White Rabbits, which were chosen for their large size and well developed ear veins. For each antigen a rabbit was immunised with the above heat-treated suspensions by injection via the marginal ear vein as follows:

Day 1	0.5 ml bacterial suspension	
Day 5	1.0 ml	"
Day 10	2.0 mls	"
Day 15	2.0 mls	"
Day 20	2.0 mls	"

On day 25, 40 mls of blood were removed form the marginal ear vein and used for the preparation of serum. On day 35 the rabbit was exanguinated under terminal anaesthesia (Animal work was performed under Home Office licence PPL 70/03323).

For the preparation of sera, red cells were allowed to clot at room temperature prior to overnight incubation at 4 °C to shrink the resultant blood clot. Sera were decanted, and the yields from both bleeds were pooled and preserved by the addition of merthiolate (0.1 mg/ml). All antisera were absorbed with a reference strain which did not express LPS to remove antibodies binding nonspecifically to core-LPS. This was done by mixing an excess of bacteria with each antiserum. The rough strain (reference strain CA385) was grown in peptone

water (37 °C, overnight), and 200 µl of bacterial suspension spread over each of seven 15 cm diameter agar plates. These plates were incubated overnight at 37 °C and the cell harvest mixed with 5 mls of antiserum for 2 h at 50 °C. Bacteria were sedimented by centrifugation at 2000 *g* for 30 min and the antiserum removed. These sera were used in agglutination reactions with both the homologous strain, used to prepare a respective serum, and for reactions against heterologous strains to detect any antigenic cross-reactions. Initially the sera were tested in microtitre plates against broth cultures of all available reference strains. The individual serum titre against its homologous O-antigen was determined in WHO plates; doubling dilutions of serum (starting at 1/50) were prepared, equal volumes of broth culture was added. Both microtitre and WHO plates were placed at 50 °C for 18 h. The plates were examined for agglutination; the titre was the highest dilution at which agglutination was seen. Having determined the titre of the serum against its homologous O serogroup, sera were stored at -10 °C until required.

When sera were found to cross-react with strains of *V. cholerae* expressing heterologous somatic antigens, sera were absorbed with the cross-reacting strain to produce specific or "pure" antisera. The absorbing strain was prepared and harvested as described above, except two 15 cm agar plates were used for each cross-reacting strain. The absorbed or pure antiserum was tested against the cross-reacting strains and the homologous strain used to prepare the serum, to ensure the specificity of the serum prepared to the homologous strain.

A single serogrouping scheme can be applied to strains of both *V.cholerae* and *V.mimicus*. Some serogroups are known to cross-react, indicating that they share certain factors in common. Known reciprocal relationships are: O2 - O9; O13 - O29; O15 - O25; O22 - O139; O23 - O73; O27 - O109; O32 - O68; O34 - O75; O65 - O80; O79 - O83; O92 - O140; O110 - O128; O115 - O126; O127 - O129 (Shimada *et al.* 1994).

2.2.4 Phage typing

A phage typing scheme has been developed for *V.cholerae* O1 (Drozhevkina and Artuyunov 1979; Bertram 1990), and strains of *V. cholerae* non-O1 were initially examined for sensitivity to phages termed "USSR classical phage 3" and "USSR El Tor phage 4". For certain strains, the full bacteriophage typing scheme, of seven USSR phages (1 -7) and three phages (designated phage 9, 10 and 11) isolated by G.Bertram, was used.

Phage typing was performed by flooding nutrient agar plates with a 3 h bacterial culture (in nutrient broth) of a given test strain, and phages were spotted onto the resultant lawn of bacteria using a Lidwell phage-typing machine. The plates were examined for lysis after incubation overnight at 37 °C, and the phage type determined by referring to patterns produced by strains of known phage type (Table 12, p103).

2.2.5 Antibiotic resistance typing (R-typing)

The resistance of bacteria to selected antibacterial agents can be used for strain characterisation. For the purpose of R-typing, nutrient agar plates were flooded with a 3 h bacterial culture (in nutrient broth) of a given test strain, and strips or discs, impregnated with antibiotics (see 2.2.5c, below) were placed on the plate prior to incubation at 37 °C, overnight. The sensitivity or resistance of a given bacterial strain to a panel of antibacterial agents was determined by the presence (sensitive) or absence (resistant) of a zone of inhibition.

2.2.5a Polymyxin discs

Filter paper discs containing Polymixin B (50 IUs per disc) were obtained from a commercial source (Oxoid Ltd.). Sensitivity to polymixin was determined as described above (2.2.5).

2.2.5b O\129 discs

Filter paper discs impregnated with 2,4-diamino-6,7-diisopropyl-pteridine (O/129), were obtained from Oxoid Ltd. Discs containing two concentrations of O\129 were used; 10 µg (O129 DD14) and 150 µg (O129 DD15) per disc. Sensitivity to O\129 was tested on agar plates as described above (2.2.5).

2.2.5c Antibiotic strips and antibacterial plates

Resistance to ampicillin (A), chloramphenicol (C), colomycin (Co), ciprofloxacillin (Cx), furazolidone (Fu), gentamicin (G), kanamycin (K), streptomycin (S), sulphathiazole (Su), spectinomycin (Sp), tetracycline (T) and trimethoprim (Tm) were tested.

The diffusion method described by Anderson and Threlfall (Anderson and Threlfall 1974) was used to test antibiotics A (200 µg/ml), C (60 µg/ml), G (60 µg/ml), K (1000 µg/ml), S (500 µg/ml) and T (5000 µg/ml). Paper strips, impregnated with antibiotic at the concentration stated in parentheses, were used. Strains were streaked onto a nutrient agar plate and the antibiotic strips were placed at right angles to the bacterial inoculum across the plate. Each test plate also contained a control *E.coli* K12 strain (LEP strain 14R525), which was known to be sensitive to all of the antibiotics. The plates were incubated overnight at 37°C.

Antibacterial plates were used to test the following antibacterial agents, Co (2 µ/ml), Cx (1 µg/ml), Fu (20 µg/ml), Su (100 µg/ml), Sp (100 µg/ml) and Tm (0.5 µg/ml). The media were prepared to give the final concentration stated. Strains to be tested were diluted to 10^{-4} and spotted onto these plates. The plates were incubated overnight at 37 °C.

2.3 Characterisation of strains based on pathogenic properties

Strains of *V. cholerae* and *V. mimicus* must possess essential pathogenic mechanisms to enable these organisms to cause disease. Pathogenic mechanisms, such as the ability to produce toxins or adhesive factors, or the expression of high affinity iron uptake compunds were used to characterise strains further.

2.3.1 Toxin production

Strains of *V. cholerae* and *V. mimicus* were assayed for the ability to produce toxin(s) using Y1, HeLa and Vero cell-lines. In addition immunoassays were used to detect CT and the infant mouse test was used for the detection of ST.

2.3.1a Bacterial culture and preparation of crude toxin

Strains were grown in 10 mls of culture medium in Erlenmeyer flasks (250 mls) for 18-24 h at 30 °C or 37 °C with rotary mixing (120 cycles/min) or statically. Bacteria were sedimented by centrifugation (17,000 *g*, 30 min, 4 °C), and supernatants sterilized by millipore filtration (0.2 μm). Bacterial filtrates were stored at 4 °C. A sample of each filtrate was incubated at 100 °C for 15 min to denature heat-labile toxins. The resultant filtrates and heated samples were used for both tissue culture tests and immunoassays. All tests were done in duplicate and the results were compared with control strains *V. cholerae* O1 (E51116 CT+) and *E. coli* (B7A LT+).

To assess optimal conditions for CT production, culture filtrates were prepared after growing the organisms in various media; Syncase sucrose broth (SSB) (Finkelstein *et al.* 1966), Syncase glucose broth (SGB) (O'Brien *et al.* 1984), Trypticase Soy broth (TSB) (Donta *et al.* 1974), Brain Heart Infusion broth (BHI) (Nishibuchi and Seidler 1983) and Casamino yeast extract broth (CYE) (Craig *et al.* 1981) (Appendix 1).

Small-scale toxin preparation

For the testing of large numbers of bacterial strains, a simpler method of toxin detection was devised requiring small volumes of medium and no Millipore filtration. The resultant preparations were used in the GM1-ELISA or VET-RPLA. For this rapid method of toxin detection, bacteria were grown in 1 ml volumes of SS broth in universal bottles (120 rpm, 37 °C). Broth cultures were transferred to sterile microcentrifuge tubes and bacteria sedimented using a microcentrifuge (12,000 *g*, 10 min). Supernatants were removed and any residual bacteria were killed with O\129 (150 μg/ml).

2.3.1b Tissue culture cell assays

Three cell lines, Y1, Vero and HeLa (Appendix 1) were used to examine bacteria for the ability to produce toxins.

Maintenance of cell lines and preparation of cell-tests

Cell-lines were maintained by regular sub-culturing, and cells were used following growth for a standard period of time for toxin tests. For subculturing; cell monolayers were initially washed (x2) with Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS). 1 ml of 0.125% (w/v) trypsin in 0.02% (w/v) versene buffer was added and poured off after 1 min, and monolayers incubated at 37 °C until cells became detached (*ca.* 5 min). Cells were suspended in 5 mls of growth medium containing foetal calf serum (Appendix 1) and 1.5 mls of cell suspension was added to 12 mls of growth medium in a tissue culture flask (75 cm^2) and incubated at 37 °C. This procedure was carried out twice weekly.

Cells required for toxin testing were obtained, when cell lines were being subcultured, and distributed to 96-well tissue culture plates (Falcon Micro Test II). The cells were diluted in tissue culture medium (TCM) to obtain a final concentration of 2.5 x 10^5 cells /

ml. In practice an approximation to this required concentration was obtained by mixing 1 ml of resuspended cells with 20 mls TCM. 200 µl of this diluted suspension was distributed in each well of a 96 well tissue culture plate. Each plate was sealed with pressure sensitive film (Falcon) suitable for tissue culture plates and incubated at 37 °C until growth was confluent, usually 3 days.

Y1 cell assays for CT and LT

The Y1 mouse adrenal cell test (Donta *et al.* 1974) was used, in which the presence of CT or LT results in morphological changes in the shape of the Y1 cells.

A monolayer of Y1 cells which had been growing for seven days was resuspended after trypsin-versene treatment and test plates prepared as described above. Toxin was assayed for by adding 25 µl of culture supernatant to duplicate wells, alongside 25 µl of heated preparations. Plates were resealed and incubated for 24 h at 37 °C. Morphological changes were observed by removing the culture medium and fixing cells with methanol (5 min) and staining with Giemsa's stain (5% v/v in phosphate buffer) for 45 min. Stained cells were washed with distilled water and plates dried prior to examination by light microscopy. Monolayers showing signs of rounding in more than 90% of cells indicated the presence of CT or LT. Heated preparations were used for comparison to ensure that cell rounding was due to a heat-labile factor.

Vero and Hela cell assays for cytotoxins

Cells were split and seeded into microtitre plates as described for Y1 cells. Filtrates were prepared and tested for cell rounding (cytotonic effect) and cell death (cytotoxic effect), except that the plates were incubated for 1 day or 3 days and then stained.

Neutralisation assays

Neutralisation tests with specific antisera prepared to CT, LT and VT were performed to confirm the specificity of the cell effects described above. The basis for the neutralisation test was that antibody binding to specific epitopes on toxin molecules, inhibits the ability of toxin molecules to function correctly. For these tests, 25 µl samples of filtrate were placed in wells of a 96-well tissue culture plate and incubated with 25 µl antiserum (diluted 1/200 in TCM) in 200 µl of TCM for 3 h at 37 °C. The contents of each well was then transferred to the respective well in a 96-well plate containing a given cell monolayer.

2.3.1c Infant mouse assay for heat-stable enterotoxin (STa)

The infant mouse test was first described by Dean *et al.* for detecting *E. coli* STa (Dean *et al.* 1972). As STa and NAG-ST are closely related the same test was used to detect NAG-ST. The work was performed under Home Office Licence PPL 70/03323. For each test two infant Schofield mice were used. The culture filtrate was prepared as described in 2.3.1a. To 0.5 ml of filtrate, 0.04 ml Pontamine blue (2% w/v) was added in order to facilitate the intragastric injections. A 0.1 ml portion of filtrate containing Pontamine blue was injected directly into the stomach, through the abdominal wall. After 4 h at 30 °C the mice were killed with chloroform and the intestine examined for distension. The ability of strains to produce toxin was determined by weighing the intestines and mice from each group of mice, a intestine weight/body weight ratio of greater than 0.1 was considered positive for STa and a ratio of less than 0.08 was considered negative. Any tests which gave a ratio between 0.08 and 0.1 were repeated. Tests were also repeated if the ratio failed to confirm the visual observation.

2.3.1d Enzyme Linked Immunosorbent Assays for CT

GM1-ELISA.

The GM1-ELISA was based on the methods described by Svennerholm & Holmgren and Sack *et al.* (Svennerholm and Holmgren 1978; Sack *et al.* 1980), and depends on the ability of the GM1 ganglioside to bind CT. ELISA plates (Dynatech Immulon) were coated with 100 µl of a preparation of ganglioside GM1 (2 µg/ml, Supelco) in PBS, covered by a plastic film and incubated at room temperature overnight. Unbound ganglioside GM1 was removed by washing plates (x3) with PBS containing 0.05% (v/v) Tween 20 (PBST). To avoid non-specific binding of toxins or antibodies, each well was blocked by adding 200 µl of 1% (w/v) bovine serum albumin (Sigma) dissolved in PBS (30 min, 37 °C). Plates were washed (x3) with PBST prior to the addition of 100 µl culture filtrate to duplicate wells and incubation at room temperature for 2 h. After washing (x3) with PBST, 100 µl of the appropriate antiserum was added. Antisera were diluted 1/200 in PBS and comprised a rabbit- anti-CT (LEP), goat-anti-CTB (Novabiochem Ltd) or rabbit-anti-LT (LEP). The plates were covered with a plastic film and left at room temperature overnight. After three washes with PBST, wells received either 100 µl of a goat-anti-rabbit IgG antibody conjugated with alkaline phosphatase for detecting binding of antibodies to CT or LT, or a rabbit-anti-goat IgG antibody conjugated with alkaline phosphatase, for detecting goat anti-CTB antibodies. Antibody conjugates were diluted 1/1000 in PBST, and 100 µl of preparation was added to the respective wells and the plates incubated at room temperature for 2 h. Plates were finally washed (x3) with PBST and 200 µl of *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer was added and plates incubated at room temperature, in the dark, for exactly 100 min. At this time, alkaline phosphatase activity was stopped by the addition of 25 µl 1 M NaOH to each well, and the absorbance read was read at 405 nm. A positive control filtrate was included on each plate, and consistently gave a reading of >1.0 (OD_{405}).

Double sandwich ELISA

In this ELISA antibodies prepared to LT, CT or CTB were used to coat ELISA plates. Each antibody was diluted 1/200 in freshly prepared coating buffer (1.59 *g* Na_2CO_3, 2.93 *g* $NaHCO_3$ per litre, pH 9.6). 100 µl of antibody preparation was added to each well and the plate covered with a plastic film and placed at 4 °C overnight. Plates were washed and blocked with BSA as described for the GM1 ELISA (above). An appropriate second ligand (ie: an antiserum prepared in a different animal from the first antiserum which was used to coat the plate) and the appropriate conjugate to detect the second ligand was used. Therefore, if the first ligand was anti-LT or anti-CT the second ligand was anti-CTB, detected using a rabbit-anti-goat IgG antibody conjugated with alkaline phosphatase; if the first ligand was anti-CTB then either anti-LT or anti-CT could be used as the second antibody and both of these could be detected using a goat-anti-rabbit IgG antibody conjugated with alkaline phosphatase.

2.3.1e *V. cholerae* enterotoxin and *E. coli* heat-labile enterotoxin - reversed passive latex agglutination test kit (VET-RPLA)

A reversed passive latex agglutination (VET-RPLA) kit (Oxoid) was also used for the detection of CT and LT. The test uses polystyrene latex particles which were sensitised with purified antiserum taken from rabbits immunised with purified *V. cholerae* CT. CT and *E. coli* LT have a similar antigenic structure therefore the antiserum raised against CT will react with both CT and LT.

In a standard agglutination a soluble antibody reacts with particulate antigen, for example bacterial cells. In the VET-RPLA the agglutination is said to be reversed because the antibody attached to the latex particles reacts with a soluble antigen. The latex particles play a passive role in the agglutination; the so -called "latex agglutination" results from cross-linking of the particles by specific antigen-antibody reaction.

Culture filtrates (crude antigen/toxin preparations) were prepared as described

for the GM1 ELISA (above). The test was performed in plates with "V"-shaped wells (Sterilin) according to the manufacturer's protocol. Doubling dilutions of filtered supernatants were made in two rows of wells using the diluent (phosphate buffered saline containing bovine serum albumin) provided with the VET-RPLA kit, to give a final volume of 25 µl in each well. An equal volume of latex beads was added to each well, sensitised beads (latex sensitised with specific rabbit IgG against CT) were added to the first row and control beads (latex sensitised with non-immune rabbit globulins) were added to the second. A CT positive control provided by the manufacturer was included on each plate. The contents of each well were mixed by hand agitation taking care that there was no spillage, the plate was covered with a lid and left on a dark vibration free surface at room temperature for 24 hours.

Each well was then examined for agglutination against a dark, preferably black, background. If CT or LT was present in the prepared filtrate agglutination occurred and the appearance was of a diffuse layer on the base of the well. If the toxin was absent or at a concentration below the assay detection level (the sensitivity of the test kit was 1 - 2 ng/ml of CT), no cross-linking occurred and a tight button was seen. Results in the row of wells containing control beads should all be negative.

2.3.1f Passive immune haemolysis (PIH)

The PIH test was originally developed for the detection of the heat-labile enterotoxin (LT) of enterotoxigenic *E. coli* (Evans and Evans 1977) and later used to detect the cholera-like enterotoxin of *V. cholerae* non-O1 (Yamamoto *et al.* 1981). Passive immune haemolysis is the complement-mediated lysis of LT-sensitised sheep red blood cells (SRBC) by antitoxin. The direct assay for LT using PIH described by Evans utilises the fact that SRBC are readily sensitised by LT in culture filtrates. The presence of toxin in the filtrates is detected when complement and antitoxin are added causing haemolysis of sensitised SRBC. Similarly CT can be detected by PIH.

Stock SRBC were obtained commercially (Tissue Culture Services Ltd) and washed twice with PBS prior to dilution in PBS to give a preparation 10% of the original concentration. Immediately before use, this preparation was diluted 1/10 in PBS. Lyophilised guinea pig complement (Gp-C') (Tissue Culture Services Ltd), was resuspended in 2 mls PBS and stored in aliquots of 0.5 ml at -10 °C. For use in the PIH test the Gp-C' was diluted 1/10 dilution in PBS. The anti-toxin was rabbit anti-CT (LEP) used at a 1/100 dilution in PBS.

Tube assay.

40 µl PBS, 10 µl toxin preparation (filtrate made as described in section 2.4.2) and 50 µl of SRBC were mixed together in a tube and incubated with shaking at 37 °C for 30 min in order to allow CT if present to adsorb to the SRBC. 50 µl of antitoxin (1/100) was then added and the mixture was incubated for an hour at 37 °C to allow the antibody to react with the CT-SRBC complexes. Next 50 µl of GP-C' (1/10) was added and the tubes were returned to 37 °C for a further hour. The reaction mixture (0.2 ml) was diluted tenfold by the addition of 1.8 mls PBS. After centrifugation at 200 *g* for 10 min to sediment the remaining SRBC the haemoglobin concentration in the supernatant was determined at 420 nm with PBS in the reference cell.

Microtitre assay.

Exactly the same reagents were used as in the tube assay, above. Two-fold dilutions of 25 µl portions of crude toxin preparation (filtrate) were performed in PBS in a U-well microtitre plate. 25 µl of washed 1% SRBC were added and the plate was incubated at 37 °C for 30 min to allow adsorption of CT if present. 25 µl of antitoxin (1/100) were added and the plate incubated for a further 30 min at 37 °C to allow the antibody to react with any CT-SRBC complexes present. Finally 25 µl Gp-C' (1/10) were added and after incubation for 1 h at 37 °C the haemolysis was read visually. The titre recorded as the highest dilution eliciting a

greater than 50% haemolysis of SRBC.

2.3.1g Biken (modified Elek) test

The Biken test was described by Honda *et al.* (Honda *et al.* 1981); this test is based on the principles of the Elek test and Ouchterlony double-gel diffusion test. Bacteria growing on Biken agar release LT which reacts with a specific antiserum placed in a well to give a line of precipitation. The formation of the precipitin line between the test colonies and anti-LT could be read by eye or observed using a light-box.

The strains to be tested were inoculated on Biken agar plates (15mls Biken agar containing 0.1 ml lincomycin (13.5 mg/ml) solution was used per plate, see appendix 1). Four strains were inoculated on each plate and incubated for 48 h at 37 °C, to obtain four large "colonies" grouped about a central site where a well was punched. The inner edge of the final growth was about 4mm from the central well position. After 48 h the central well was punched and a polymixin B disc (500 units) was placed on top of each colony. The plate was incubated for 5 h and then 20 µl of rabbit-anti-CT or rabbit-anti-LT, was added to the central well and the plate was incubated for 24 h at 37 °C. The plate was then examined in a light box for precipitin lines. The precipitin lines could be enhanced by a further 24 h incubation.

2.3.1h Detection of toxin with immunoblotting

Colony immunoblotting

Test strains of *V. cholerae* were grown in nutrient broth and spotted onto nutrient agar plates in a grid pattern (a maximum of 25 strains) prior to incubation at 37 °C for 6 h. Positive (WBDV-101E O49,CT+; E51116 O1,CT+) and negative controls (*E. coli* LEP 14R519) were included on each membrane. A nitrocellulose membrane (Hybond C-extra), marked to indicate membrane orientation, was placed onto the network of bacterial colonies,

and the membrane gently rubbed with a glass spreader to ensure contact between the membrane and the bacterial colonies. After 5 min membranes were peeled from the agar plate and placed in plastic boxes containing 5% (w/v) dried milk powder in PBS, blocking was maximised by shaking boxes for 30 min. Membranes were transferred to plastic bags, up to 8 membranes per bag, containing 20 mls of fresh blocking solution. Antiserum was used at a dilution of 1/200 in blocking solution and both anti-CT (LEP) and anti-CTB (Novabiochem) were used. Bags were heat-sealed and incubated, with shaking, for 2 h at room temperature, after which they were transferred to a plastic box and washed (3 x 10 min) in PBS. Antibodies binding to toxin molecules were detected with an alkaline phosphatase conjugated goat anti-rabbit immunoglobulin for rabbit antibodies to CT, diluted 1/7000 in blocking buffer. Similarly, an alkaline phosphatase conjugated rabbit anti-goat immunoglobulin for goat-anti-CTB antibodies, diluted 1/3000 in blocking buffer. Membranes were mixed with conjugated antibodies in polythene bags for 2 h. Membranes were washed for 3 x 10 min in PBS and rinsed briefly in alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). The substrate solution was 69 µl NBT (75 mg/ml nitroblue tetrazolium in 70% dimethyl formamide) and 54 µl BCIP (50 mg/ml 4-bromo-3-chloro-indolyl phosphate in water) to 15 mls buffer (this was sufficient for eight membranes). Dark purple spots developing in 5 min indicated a positive reaction, indicating the presence of toxin. The reaction was stopped after 30 min or as desired by washing in water.

Immunoblotting with supernatants

This method was essentially the same as the colony immunoblotting, except that supernatants were used. It is a modification of the method of Qu *et al.* (Qu *et al.* 1991). The strains to be tested were grown in syncase sucrose broth as described in section 2.3.1a. 10 µl of the undiluted supernatants were spotted onto nitrocellulose (Hybond C-extra) membranes. The blocking procedure, addition of antiserum and detection were as described above.

2.3.2 DNA probes for genes encoding toxins

Polynucleotide and oligonucleotide probes were used to detect the genes for CT and ST. Polynucleotide probes were used to detect VT genes. Details of polynucleotide probes used are given in table 4 and details of oligonucleotide probes are given in table 5.

2.3.2a Large-scale preparation of plasmid DNA

The method used for the production of large amounts of plasmid DNA was based on the method of Birnboim and Doly (1979) as described by Maniatis *et al.* (Maniatis *et al.* 1982). Strains of *E.coli* K12 carrying plasmids with cloned gene sequences were used to prepare large quantities of covalently closed circular DNA.

E.coli K12 strains containing plasmid DNA were grown in 50 mls of nutrient broth at 37 °C overnight with shaking. These starter cultures were added to 450 ml volumes of nutrient broth in 2 litre conical flasks and grown with shaking for 1 - 2 h until log phase growth was reached. The optical density of cultures was read immediately after inoculation, using nutrient broth as a blank in a Klett-Summerson Photoelectric Colorimeter. When the bacterial cell density attained an optical density of 140 Klett units, each culture was split into 3 equal volumes and the cells sedimented at 16,000 *g* for 5 min. Cells from each batch were resuspended in 10 mls of 50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA and 50 mg of lysozyme, and 5 mls was placed into two 50 mls centrifuge tubes. Following incubation at room temperature for 5 min, 10 mls of freshly made 1% SDS (sodium dodecyl sulphate) in 0.2N NaOH were added to each tube and mixed thoroughly by inversion. 7.5 mls of ice-cold 5 M potassium acetate, pH 4.8 were added, mixed and the tubes placed in ice for 10 min. Cell debris was precipitated by centrifugation (38,000 *g*, 20 min at 4 °C) and supernatants pooled in a 100 mls measuring cylinder. 0.6 volumes of isopropanol were added and mixed well. The mixture was divided equally between two poly-carbonate centrifuge tubes and placed at 4 °C overnight. Precipitated plasmid DNA was sedimented by centrifugation (8,000 *g*, 20 min at

Table 4: Bacterial strains and plasmid vectors containing polynucleotide probe DNA.

Polynucleotide probe	Strain	RE used to prepare probe	Reference
CTA 554 bp	*V. cholerae* 62746 cloned into *E. coli* K12 pBR325 (pCVD27)	*Eco*RI	Kaper, 1981
CTB 550 bp	*V. cholerae* 1621 cloned into *E. coli* K12 pACYC184 (pCT19)	*Xba*I, *Hin*cII	Gennaro, 1982
VT1 750 bp	*E. coli* 60R746 NTP705 (pACYC177)	*Hin*cII	Willshaw, 1985
VT2 850 bp	*E. coli* 62R310 pDEP28 (pGEM1)	*Sma*I, *Pst*I	Thomas, 1991

Table 5: Oligonucleotide probe sequences.

Oligonucleotide	Probe sequence	Reference
GM1 24 bases	Mixed CTB and LTB sequences	Calva, 1989
	CTB 5' TTT ATT ATT CCA TAC ACA TAA CTT 3'	
	LTB 5' TTT GTT GTT CCA TAC ACA TAA TTT 3'	
STI 23 bases	5' GAT TGC TGT GAA ATT TGT TGT AA 3'	Hoge, 1990
STII 24 bases	5' GAC TGC TGC GAA ATA TGT TGC AAT 3'	Ogawa, 1990

room temperature). The supernatant was discarded and the pellet was washed in 70% ethanol. As much of the ethanol as possible was removed and the pellet was resuspended in 4 mls of Tris EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8). The DNA/TE solution was transferred to a pre-weighed 10 mls polycarbonate centrifuge tube and the weight made up to 5 g with TE buffer. To this was added 5.2g of caesium chloride and 0.4 ml of 10 mg/ml ethidium bromide. Tubes were centrifuged in a Beckman L8-70M centrifuge at 16,000 *g* for 30 min and the supernatant was transferred to 10 mls polyallomer tubes. The density of caesium chloride was checked by measuring the refractive index using a refractometer. The refractive index of the solution was adjusted to 1.395 by the addition of caesium chloride (if too low) or water (if too high). The tubes were capped and paraffin was added to fill up the tube avoiding air bubbles. Grub screws were fitted and the tubes weighed to check the balance of the pair. The gradient was formed by centrifugation in a Beckman or MSE65 centrifuge at 195,000 *g* at 20 °C for 60 h.

Tubes were examined under UV light (385 nm) to visualise the two bands. The upper band was open closed circular plasmid DNA and linear (chromosomal) DNA, while the lower band contained the covalently closed circular DNA which was recovered. The lower band of DNA was carefully removed from the tube by inserting a syringe needle through the side of the tube. Ethidium bromide was extracted from closed circular DNA by adding an equal volume of isopropanol, shaking and removing the upper layer containing the ethidium bromide. This procedure was repeated two or three times to ensure all the ethidium bromide was removed. The DNA was dialysed in 2 litre of TE buffer at 4 °C for 6 h to remove any contaminating ions. The size and purity of the plasmid DNA was checked by agarose gel electrophoresis. Plasmid DNA was stored at -20 °C until required.

2.3.2b Preparation of polynucleotide probes.

To prepare probes for use in hybridisation experiments, probe fragments were digested from plasmid vectors using restriction enzymes.

Recovery of CT fragments.

The CT gene sequence from *V.cholerae* El Tor 62746 (Kaper and Levine 1981) cloned into plasmid pBR325 (pCVD27) in *E.coli* K12 was provided by J.B.Kaper (Kaper and Levine 1981). A 554 bp CTA probe fragment was cut from pCVD27 after digestion with *Eco*RI (BRL): three aliquots of 40 µl of pCVD27 were digested with 20 units of *Eco*R1 (Gennaro *et al.* 1982).

The recombinant plasmid pCT19 is known to contain sequences encoding CTB and part of CTA (Dallas and Falkow 1979); the gene sequences were cloned and subcloned from *V.cholerae* 1621 into pACYC184 as described by Gennaro *et al.* (Gennaro *et al.* 1982). A 550 bp CTB probe fragment was cut from plasmid pCT19 after digestion with *Xba*I and *Hin*cII: three aliquots of 40 µl of pCT19 were digested with 20 units each of *Xba*I and *Hin*cII.

Probe fragments were separated by electrophoresis and on a 1% agarose gel in Tris acetate buffer at 20 V overnight. A lambda *Pst*1 molecular weight marker was run on the same gel. The gel was stained in ethidium bromide and fragments were cut out of the gel under long wave UV light into a preweighed screw cap Eppendorf tube. Four or five gel slices, weighing in total between 0.4 and 0.5 g, were pooled and the DNA was recovered from the gel and purified using the Geneclean Bio 101 kit (Stratech Scientific Limited) following the method provided by the manufacturer. A volume of sodium iodide (three times the weight of the slices) was added to the gel. The gel was dissolved by incubation at 50 °C for 2 min. If the amount of DNA was unlikely to exceed 5 µg, 5 µl glass milk were added to bind the DNA by placing on ice for 5 min. The DNA bound to the glass milk, was washed three times by pelleting the glass milk for 5 sec, followed by resuspension in 500 µl NEW wash buffer

(supplied by the manufacturer; Tris/EDTA, NaCl, ethanol, water). The DNA was then eluted by resuspending the pellet in 5 µl TE at 50 °C for 3 min, followed by centrifugation for 30 sec. This was repeated twice to give a total of 15 µl DNA. A volume of 1 µl was run on an agarose gel to check its size and concentration.

Recovery of VT fragments.

The VT1 DNA probe is a 750 bp *Hinc* II fragment from plasmid NTP705 (Willshaw *et al.* 1985), and the VT2 probe is a 850 bp *Sma* I - *Pst* I fragment from plasmid pDEP28 (Thomas *et al.* 1991). The fragments were separated on an agarose gel and probe DNA excised as described above.

2.3.2c Random primer labelling of polynucleotide DNA probes with digoxigenin.

The cloned polynucleotide probes were labelled with digoxigenin, based on the protocol provided with the Digoxigenin labelling and detection kit (Boehringer Corporation Ltd). 1 µl of purified DNA was run on a gel to check size and intensity. Increased yields of labelled probe could be obtained by increasing the volume of the labelling reagents, whilst keeping the amount of DNA the same.

Digoxigenin-labelling of CT probes.

7.5 µl of purified DNA were denatured by boiling for 10 min followed by rapid cooling on ice, 5 µl hexanucleotide primers (2 mg/ml), 5 µl nucleotides (1 mM dATP, 0.65 mM dTTP, 1 mM dGTP, 1 mM dCTP and 0.35 mM digoxigenin-11- dUTP) and 2.5 µl Klenow polymerase enzyme (2 U/µl) were added. Labelling took place overnight at 37 °C. The reaction was stopped by adding 5 µl 0.2 M EDTA (pH 8).

To remove unincorporated nucleotides the labelled probe was precipitated and then cleaned using a QIAGEN-tip 5 (DIAGEN, Dusseldorf). The precipitation step involved

adding 2.5 µl 4 M LiCl and 75 µl prechilled ethanol and mixing well. After a minimum period of 2 h at -20 °C, the probe was centrifuged at 10,000 *g* for 5 min. The pellet was washed with 70% chilled ethanol and centrifuged. The ethanol was removed and the pellet allowed to dry. The probe was resuspended in 50 µl TE containing 0.1% SDS. To use the QIAGEN-tip 5, the volume was made up to 150 µl with distilled water. 18 µl 5 M NaCl and 10 µl 1 M MOPS was added. The QIAGEN tip was equilibrated using kit buffer A (see appendix 1), 500 µl were allowed to pass through by gravity flow. The labelled probe suspension was loaded onto the matrix and allowed to pass through under gravity. The matrix was washed with 2 mls kit buffer B. The probe was eluted with 600 µl of kit buffer F and collected in a microcentrifuge tube. The total volume was typically 800 µl. 10 µl 20 mg/ml yeast tRNA was added as a carrier molecule, followed by 0.6 x volume of isopropanol. After precipitation at 4 °C, the probe was centrifuged at 10,000 *g* for 10 min and resuspended in 100 µl TE/0.1% SDS. Labelled probe was stored at -20 °C.

Digoxigenin-labelling of VT probes.

In a standard labelling reaction 3 µl of purified DNA were used. The DNA was made up to 15 µl with distilled water, boiled for 10 min to denature and cooled rapidly on ice. The following were added; 2 µl hexanucleotides, 2 µl nucleotides (dATP, dTTP, dGTP, dCTP and digoxigenin-11-dUTP), 1 µl Klenow polymerase giving a total volume of 20 µl. Labelling took place overnight at 37 °C overnight. The labelling reaction was stopped by adding 2 µl 0.2 M EDTA. Unincorporated nucleotides were removed as described and the labelled probe was stored at -20 °C.

2.3.2d Preparation of cultures for DNA hybridisation.

Bacterial cultures, grown overnight in nutrient broth, were spotted onto nylon membranes (Hybond N Amersham) placed on nutrient agar plates. Colonies were grown at 37

°C for 6 h, the cells were then lysed and DNA denatured by the following procedure. The membranes were laid onto 3 mm blotting paper soaked in 10% SDS for 10 min; 0.5 M NaOH/ 1.5 M NaCl for 20 min; neutralising solution (1.5 M NaCl/ 0.5 M Tris HCl, pH 8) for 10 min and 2 x SSPE for 10 min. DNA was fixed to the membrane by baking for 2 h at 80 °C.

2.3.2e Hybridisation with polynucleotide probes.

Hybridisations were performed either in heat-sealed plastic hybridisation bags placed on a shaker in a water bath or in a hybridisation oven. Small scale experiments were performed to select the appropriate temperature and optimise conditions. (Detection procedures are described in 2.6.7). The hybridisation procedure was essentially the same for water-bath or oven (both are described below).

To use a water bath the membranes were soaked in 2 X SSC then placed in a plastic box and covered with hybridisation solution (approximately 20 mls per 100 cm^2 membrane). The box was shaken for 1 h at the hybridisation temperature. After 1h the membranes were gently wiped with a damp tissue to remove cellular debris. The membranes were replaced in the box for pre-hybridisation with a freshly prepared solution, containing 10 µl/ml denatured salmon sperm DNA, for a further 1 - 2 h with shaking. The probe to be used and 10 µl/ml salmon DNA, were denatured by boiling for 5 - 10 min and placed on ice. The membranes were placed in heat-sealable plastic bags, ensuring that they did not overlap. For every 100 cm^2 of membrane, 2.5 mls of hybridisation solution containing 26 ng/ml of labelled denatured probe (typically 5 - 10 µl) were added. The bags were sealed and placed in another bag in the water bath. Hybridisation was carried out overnight with shaking at the appropriate temperature. The probe was removed and stored at -20 °C for further use. The membranes were placed in a box for washing.

To use a hybridisation oven, the oven was pre-heated to the hybridisation temperature with the safety thermostat set at a few degrees above. Pieces of HYBAID nylon

mesh were cut according to bottle size and number of membranes to be tested. Both the mesh and the membranes were soaked in 2 X SSC. The membranes were placed on the mesh, ensuring there was no overlap, and rolled up. The roll was placed in the bottle and enough hybridisation solution added to fill about 1/4 of the bottle. The bottle was gently rolled to unwind the membranes, before placing in the preheated oven. After 1 h rotation, membranes were wiped and pre-hybridised for a further 1 - 2h, followed by hybridisation overnight. Smaller volumes (2 - 5 mls) of hybridisation solution and probe could be used in the oven as good contact was achieved with the membranes. After removal of the probe solution washes could be carried out with the membranes still in the bottle.

Hybridisation with CTA and CTB at high stringency.

Hybridisation was carried out at 68 °C, with stringent washing conditions which allowed the detection of DNA sequences with more than 80% homology.

The hybridisation solution was; 5 x SSC, 0.1% sodium-laurylsarcosine, 0.02% SDS, 1% blocking reagent (BCL). Hybridisation procedures were as described, above. Post-hybridisation washes were 2 x SSC/ 0.1% SDS for 5 min at room temperature followed by two washes of 15 min each with 0.1% SSC/ 0.1% SDS at 68 °C. Detection was carried out as described by BCL. The probe could be saved and reused.

Hybridisation with CT probes at low stringency.

Selected *V.cholerae* non-O1 strains were also tested under lower stringency conditions (37 °C), which allowed the detection of both CT and *E.coli* LT. The protocol for hybridisation at 37 °C was as described above, however the hybridisation solution was 25% formamide/ 5 x SSC/ 1 x Denhardts solution/ 0.1% SDS/ 1 mM EDTA. The post-hybridisation washes were 5 x SSC/ 0.1% SDS at 54.5 °C briefly and then for 1 h, followed by 2 x SSC at room temperature briefly. Detection was carried out as described by BCL. The probe could be saved and reused.

Hybridisation with VT1 and VT2 probes.

The hybridisation temperature was 68 °C. The hybridisation solution and post-hybridisation washes were as described for high stringency CTA and CTB. Detection was carried out as described by BCL. The probe could be saved and reused.

2.3.2f Synthetic Nucleic Acid Probe for LT (SNAP-LT) and STA (SNAP-ST)

Alkaline-phosphatase labelled enzyme-linked oligonucleotide probes were obtained from DuPont NEN Products (Boston, USA). The SNAP-ST kit incorporated both STA1 and STA2 (Scotland *et al.* 1989).

The SNAP probes were used at a concentration of 50 ng/ml. The procedure was that provided by the manufacturer. Hybridisations were performed at 50 °C and the hybridisation buffer was 5 x SSC, 1% SDS, 0.5% BSA, 0.5% polyvinylpyrolidone (PVP). Post-hybridisation washes were; two 5 min washes in 1 x SSC/ 1% SDS (at 50 °C for SNAP-ST and at 40 °C for SNAP-LT), two 5 min washes in 1 x SSC/ 1% Triton (at 50 °C for SNAP-ST and at 40 °C for SNAP-LT), followed by two washes in 1 x SSC at room temperature. After the washes a direct detection was carried out by adding the substrates (15 mls alkaline phosphatase buffer, 66 µl nitroblue tetrazolium (NBT), 50 µl 5-bromo-4-chloro-3 indolyl phosphate (BCIP)). After 3 h or time for the purple coloration to appear the filters were washed in distilled water and dried between filter paper sheets. The filters were stored in plastic folders in the dark.

2.3.2g Oligonucleotide probes

Oligonucleotides were synthesised by the Virus Reference Division, CPHL, using an Applied Biosystems DNA synthesiser. The sequences were evaluated for the possibility of dimer and loop formation using OLIGO Primer Analysis Software (MedProbe, Norway).

GM1 probe

The GM1 probe was a mixed oligonucleotide based on the sequence in Calva *et al.* (Calva *et al.* 1989; Chopra *et al.* 1991):

5' TTT **A/GTT A/GTT** CCA TAC ACA TAA **C/TTT** 3'

ST probes

For the ST probes two different oligonucleotides based on the sequences of Hoge *et al.* and Ogawa *et al.* were used:

GAT TGC TGT GAA ATT TGT TGT AA (Hoge *et al.* 1990)

GAC TGC TGC GAA ATA TGT TGC AAT (Ogawa *et al.* 1990)

2.3.2h Oligonucleotide end-labelling with digoxigenin-11-dUTP

The DNA concentration (C) of each oligonucleotide synthesised on a Applied Biosystems DNA synthesiser was calculated using the formula

$$OD = e.C$$

The optical density (OD) was measured for each oligonucleotide (1 OD was equivalent to approximately 33 μg ss DNA).

The extinction coefficient (e) was the sum of the number of each base multiplied by the appropriate extinction coefficient: dGTP = 11.7 ml/ μMol; dCTP = 7.3; dATP = 15.4; dTTP = 8.8.

Single stranded probe DNA was stored in water at -20 °C. For BCL reagents the labelling reaction was: 4 μl tailing buffer, 4 μl CoCl2 (25 mM), 100 pMoles oligonucleotide, 1 μl 1 mM Dig-dUTP and 1 μl terminal transferase (50 U/μl) were mixed together in a microcentrifuge tube. The volume was made up to 20 μl with distilled water and incubated for 1.5 h at 37 °C, then placed on ice. 1 μl glycogen (20 mg/ml) was mixed with 200 μl EDTA (0.2 M pH 8), and a 2 μl aliquot added to the mixture to stop the reaction. The

labelled oligonucleotide was precipitated with 2.5 µl LiCl (4 M) and 75 µl pre-chilled ethanol, this was mixed well and left for 2 h at -20 °C. The precipitate was pelleted by centrifugation and washed with 70% ethanol. The pellet was resuspended in an appropriate volume of distilled water and stored at -20 °C. To check the labelling, 5 µl of probe were run on a 3% agarose mini-gel using borate buffer and 100 V for 1 h with Orange G as the dye marker. The probe was transferred to a nylon membrane by Southern blotting and a BCL direct detection was performed to visualise labelled probe.

2.3.2i Oligonucleotide hybridisation

The OLIGO Primer Analysis Software (MedProbe, Norway) program was used to calculate the melting temperature (Tm) from which subsequent hybridisation temperatures were derived. The hybridisation temperature was 5 - 10 °C below the Tm of the sequence and the probe concentration used was 50 ng/ml in the appropriate hybridisation buffer.

Colony blot membranes were prepared as described in section 2.3.2d. DNA was fixed to the membranes by baking at 80 °C for 2 h. The membranes were pre-washed in hybridisation solution at 50 - 65 °C for 1 - 2 h. This was followed by gentle wiping of the membranes with a damp tissue to remove cellular debris. The membranes were pre-hybridised for 1 - 2 h with hybridisation solution, and then hybridised overnight with 2 mls hybridisation solution containing probe equivalent to 50 ng/ml for each 50cm^2 membrane. All steps were performed in heat sealable plastic bags with gentle shaking, or using a hybridisation oven. The detection procedure was carried out as described by BCL. (For blocking solutions, hybridisation buffers and wash solutions see appendix 1).

2.3.2j Immunological detection of hybrids.

The detection procedure was carried out as described by BCL. All steps were carried out at room temperature on a shaker, unless otherwise stated. Fresh blocking solution

was made as described in appendix 1. The membranes were washed briefly in buffer 1, then placed in 100 mls buffer 2 for 30 min. The conjugate was diluted from 1:5000 (4 µl in 20 mls) in buffer 1, 20 mls is sufficient for 100 cm^2 of membrane. The membranes were removed from buffer 2, washed briefly in buffer 1, and placed in conjugate solution for 30 min. Membranes were transferred to a fresh box and equilibrated with buffer 3 for 2 min. Membranes were then placed in 10 mls colour solution in the dark without shaking. Once purple/brown colour appeared, membranes were rinsed with water, dried in the dark and stored in plastic folders away from the light.

2.3.3 Polymerase chain reaction (PCR)

The PCR was used to amplify *ctxB* and *tcpA* sequences in *V.cholerae*, see Figure 3a and 3b.

2.3.3a Detection of *ctxB* by PCR

Primers for *ctxB* were from Olsvik *et al.* (Olsvik *et al.* 1993):

CTX7 5' GGT TGC TTC TCA TCA TCG AAC CAC 3'

CTX9B 5' GAT ACA CAT AAT AGA ATT AAG GAT G 3'

Primer CTX7 is the antisense strand and CTX9B is the sense strand. These oligonucleotide primers target the *ctxB* sequence.

2.3.3b Detection of *tcpA* by PCR.

Primers for *tcpA* are from Keasler & Hall (Keasler and Hall 1993):

tcp1 classic 5' CAC GAT AAG AAA ACC GGT CAA GAG 3'

tcp2 classic 5' ACC AAA TGC AAC GCC GAA TGG AGC 3'

tcp3 El Tor 5' GAA GAA GTT TGT AAA AGA AGA ACA C 3'

tcp4 El Tor 5' GAA AGG ACC TTC TTT CAC GTT G 3'

Primers tcp1 and tcp3 are the sense strands, tcp2 and tcp4 are the antisense strands. These oligonucleotide primers exploit sequence differences between the *tcpA* of the El Tor and classical biotypes of *V.cholerae* O1.

2.3.3c PCR protocol

Small batches of tubes were prepared containing all components except the nucleotides which were added with the template prior to performing the PCR in the thermocycler.

Tubes were prepared in a laminar flow cabinet. Stocks of primers, dNTP's and other reagents were prepared in advance. To a sterile tube the following were added; 250 µl Bioline 10 x buffer, 75 µl MgCl2 (50 mM), 250 µl primer 1 (10 µM), 250 µl primer 2 (10 µM), 10 µl Biotaq (5 U/µl) 415 µl sterile glass distilled water. This was then aliquoted 25 µl per tube and 1 drop of Sigma oil was added to each. The tubes were stored at -10 °C. For use the required number of tubes were thawed and 5 µl dNTP mix (final concentration of each dNTP was 200 µM) was added to each tube. The culture (or template) was prepared by subculturing an overnight broth into fresh nutrient broth, and growing for 2 - 4 h. 2 µl of template were added to the PCR reaction mix and made up to the correct volume by adding 18 µl sterile glass distilled water. The cycling conditions using a Quatro TC-40I were as follows:

5 min 95 °C;

25 cycles of 1 min 95 °C, 1 min 50 °C and 1 min 72 °C;

10 min 72 °C.

2.3.3d Analysis of PCR products

Water (10 µl) and Orange G (3 µl) were added to 20 µl of product and run on a 1.5 - 3% gel (depending on expected product size; a 2% gel was appropriate for an expected product size of 450 bp). Electrophoresis was carried out in Tris-Borate buffer at 100 V for

Figure 3a: Polymerase chain reaction for CT.

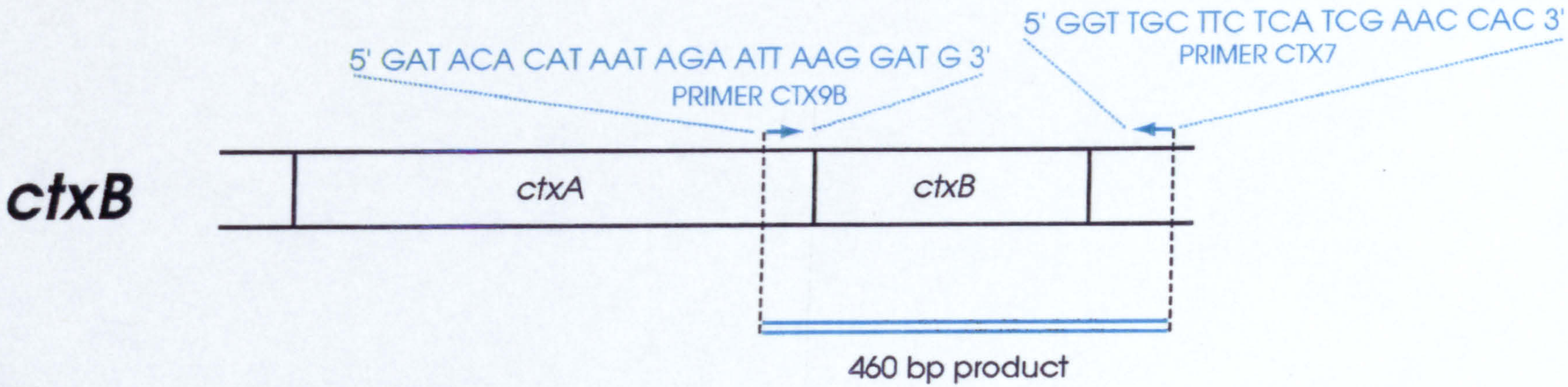
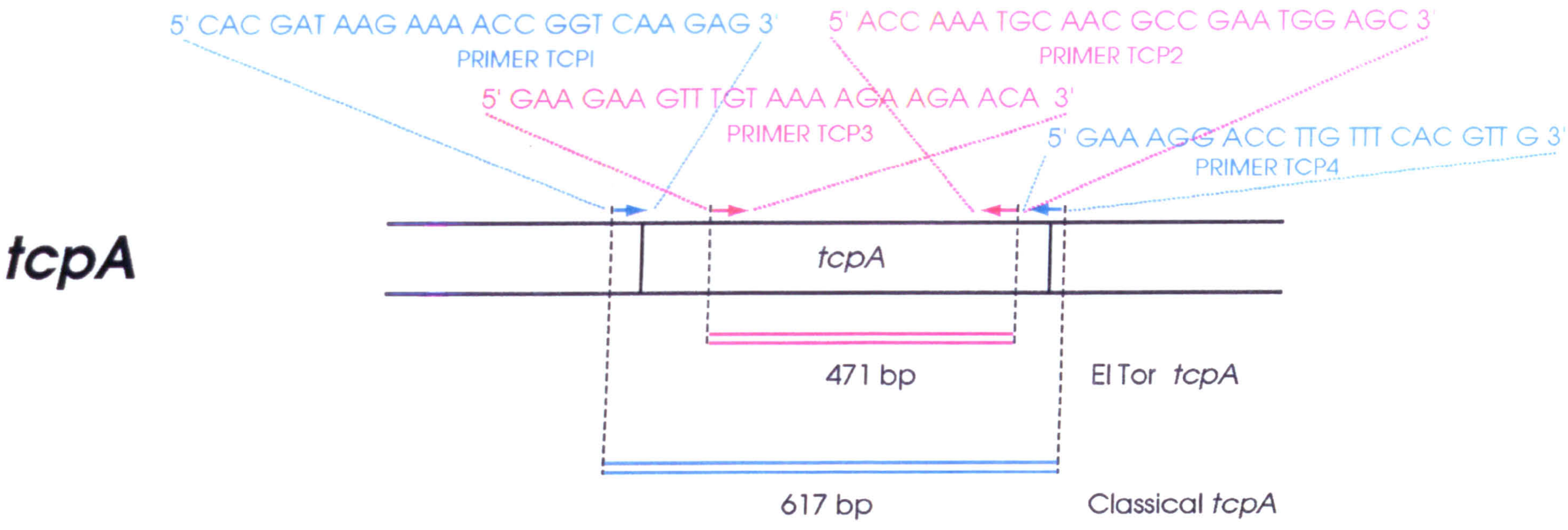


Figure 3b: Polymerase chain reaction for TCP.



2 - 3 h. The DNA was visualised by ethidium bromide (0.5 μg/ml) staining and bands confirmed by oligonucleotide probe hybridisation.

2.3.4 Preparation of chromosomal DNA and analysis by Southern blot

Chromosomal DNA was prepared from strains shown to possess the genes for CT or ST. The DNA was analysed by digesting with restriction endonucleases and Southern blotting, followed by hybridisation with the appropriate probe.

2.3.4a Preparation of bacterial chromosomal DNA

Chromosomal DNA was extracted using the method of D.Platt, as described in G.Bertram, MPhil. (Bertram 1990). Strains were grown in 3 mls nutrient broth at 37 °C overnight. 200 μl of culture were subcultured into 10 mls nutrient broth and incubated at 37 °C for 2 h with shaking. Cells were harvested by centrifugation for 10 min at 3,000 rpm in a Sorvall 6000 centrifuge and the cell pellet was washed in 1 ml of TE50 buffer (50 mM Tris-HCl, 50 mM EDTA; pH 7.8). The suspension was pelleted in an Eppendorf micro-centrifuge and the supernatant was removed. The pellet was resuspended in 0.5 ml of TE50 buffer and 50 μl of fungal proteinase K (10 mg/ml) was added to the suspension. 15 μl of SDS (20% w/v) was added, mixed gently and then incubated at 55 °C for 4 h to lyse cells. To precipitate cell debris, the suspension was emulsified with 0.5 ml of freshly prepared phenol/chloroform. The emulsion was broken by centrifugation for 10 min in an Eppendorf microcentrifuge and the upper aqueous layer was removed to a clean Eppendorf tube. To recover the maximum amount of DNA, a further 200 μl of TE50 buffer were added to the Eppendorf tube and emulsified. The emulsion was again broken by centrifugation and the aqueous layer was removed and pooled with the first supernatant. The chromosomal DNA was precipitated by adding 0.5 ml of isopropanol and vortexed. The DNA was left in isopropanol at room temperature for 10 min to allow complete precipitation. The chromosomal DNA was

pelleted by centrifugation for 10 min and the isopropanol was discarded. The pellet was resuspended in 0.3 ml of TE10 buffer (10 mM Tris-HCl, 10 mM EDTA; pH 7.8), vortexed thoroughly and left to stand at room temperature for 5 min to allow the DNA to resuspend. 100 µl of 7.5 M ammonium acetate were added and mixed. DNA was precipitated by adding 0.6 ml of ethanol (95% w/v) and placed at 4 °C overnight. The precipitate was pelleted by centrifugation in an Eppendorf microcentrifuge for 15 min. The supernatant was discarded and the DNA was resuspended in 0.3 ml of TE10 buffer and allowed to stand at room temperature for 5 min. 20 µl of heat-treated RNase (1 mg/ml, boiled for 10 min) was added and incubated at 37 °C for 1 h to remove RNA from the chromosomal preparation. The phenol/ chloroform extraction was repeated as described above and the DNA was precipitated by repeating the isopropanol and ethanol steps. The DNA was placed at 4 °C overnight, then pelleted by centrifugation for 15 min in a microcentrifuge. The supernatant was discarded and the pellet was resuspended in 50 µl of TE buffer.

2.3.4b Small-scale preparation of bacterial genomic DNA (CTAB method)

This method uses proteinase K to remove proteins and cetyltrimethylammonium bromide (CTAB) to remove cell wall debris, polysaccharides and remaining proteins. High molecular weight DNA was isolated by isopropanol precipitation.

The test organisms were grown overnight in nutrient broth. The cells were concentrated by centrifuging 1.5 ml of broth in a microcentrifuge. After resuspending in 579 µl TE, 15 µl 20% SDS and 6 µl proteinase K (10 mg/ml) were added and the mixture was placed at 37 °C. After 1 h 110 µl 5 M NaCl and 80 µl CTAB/NaCl mix (10% CTAB in 0.7 M NaCl) were added and incubated for 10 min at 65 °C. The DNA was extracted by adding equal volumes of chloroform/ isoamyl alcohol (24:1) followed by phenol/ chloroform/ isoamyl alcohol (24:24:1). The DNA was transferred to a fresh tube and precipitated by adding isopropanol at room temperature for 15 min. Then the DNA was spun for 10 min in a

microcentrifuge. There was a clearly visible pellet which was then washed with 70% ethanol. After drying the pellet was resuspended in 50 µl TE/RNase and incubated at 37 °C for 30 min. The DNA was stored at 4 °C.

2.3.4c Detection of DNA on ethidium bromide plates

This was useful as an approximate guide to how much DNA had been extracted from each strain. Ethidium bromide plates were prepared as described by Maniatis (Maniatis *et al.* 1982). 3 µl of sample DNA and standard concentrations of 5, 10, 15, 20 ,25 and 30 µg/µl of herring sperm DNA were spotted on 1% agarose plates containing 0.5 µg/ml ethidium bromide and incubated at 37 °C for 15 min. The plates were then placed under a UV transilluminator and the fluorescence of the sample DNA was compared to that of the standard concentrations.

2.3.4d Restriction endonuclease digest of chromosomal DNA

Chromosomal DNA from each strain was digested with various restriction endonucleases: *Eco*R1, *Hin*dIII, *Bgl*II and *Pst*1 (Bethesda Research Laboratories, BRL). For each digest, 20 µl of DNA were digested with 20 units of enzyme. 4 µl of the appropriate reaction buffer (supplied with the enzyme) was added and the reaction mix was incubated at 37 °C for 6 h. Digested DNA was run alongside a lambda *Pst*1 digest control in a 1% agarose gel made up in Tris acetate buffer (0.04 M Tris acetate, 0.002 M EDTA, pH 8) at 20 V overnight on a midi gel system (BRL).

2.3.4e Southern blotting of DNA from agarose gels to nylon membrane filters

After staining the gel with ethidium bromide, the gel was photographed and the sides, bottom and unwanted tracks were removed. The top of the gel was not trimmed but the top right hand corner was cut off as an orientation mark. The gel was transferred to a plastic

box and treated with 0.25 N HCl for 20 min. The gel was rinsed in distilled water, then soaked in denaturing solution for 40 min with one change of solution (500 mls was sufficient for a full-sized gel). The gel was rinsed again with distilled water and soaked in neutralising solution for 40 min with one change of solution. The gel was accurately measured and a piece of nylon membrane (Hybond N) cut to the same measurements.

The blotting apparatus was assembled as follows: a plastic box was used as a reservoir; it containing the blotting solution (6 x SSC). An appropriately sized glass plate was placed over the reservoir and the wicks, two sheets of 3 MM paper which reached the bottom of the reservoir, were placed on top of the glass plate. The gel was placed on the wet wicks avoiding air bubbles or stretching the gel. The gel surface was moistened with 6 x SSC and the nylon transfer membrane placed on the gel, this was gently smoothed out to remove any air bubbles. A blotting stack was built up, starting with 6 - 8 sheets of 3 MM paper cut to exact size, and continuing with absorbent paper towel, folded or cut, to fit accurately. A thick glass plate was placed on top and plastic film was wrapped round to retain moisture. The blot was left overnight. The next day, the apparatus was taken apart and the right side of the nylon membrane marked in pencil. The DNA was fixed by baking at 80 °C for 2 h.

2.3.4f Restriction fragment length polymorphism (RFLP) analysis.

The Southern blots were hybridised with the appropriate probe as described in section 2.3.2. The probes used for RFLP were polynucleotides CTA+B and oligonucleotides GM1 and ST.

2.3.5 Plasmid profile analysis

DNA was extracted by the methods of Kado and Liu (1981) or Birnboim and Doly (1979) (Birnboim and Doly 1979; Kado and Liu 1981).

2.3.5a The phenol/chloroform extraction method (Kado and Liu)

Cultures were grown in 3 mls of nutrient broth at 37 °C. 1.5 mls of culture were centrifuged for 2 min in a microcentrifuge. The supernatant was discarded and the cell pellet resuspended in 20 µl of 50 mM Tris /1 mM EDTA, pH 8. 100 µl of 0.05 mM/ 3% SDS pH 12 was added and mixed with care. The samples were incubated at 55 °C for 45 min. 100 µl of freshly made phenol/chloroform (50% w/v phenol in chloroform isoamyl alcohol (24:1) saturated with 10 mls of 10 mM Tris-HCl, pH 8 containing 1 mM EDTA) was added and emulsified by vortexing thoroughly. After centrifugation for 10 min to precipitate cell debris, the upper aqueous layer was transferred to a fresh Eppendorf tube. The precipitation was repeated by adding a further 100 µl of phenol/chloroform. The samples were centrifuged for a further 10 mins and the upper layer was removed to a fresh Eppendorf tube. 7 µl of bromophenol blue was added to 40 µl of supernatant and loaded onto a 0.8% agarose gel made up in Tris borate buffer (0.089 M Tris, 0.089 M Boric acid, 0.008 M EDTA) and electrophoresed at 120 V for 3 h.

2.3.5b The alkaline lysis method (Birnboim and Doly)

As recovery of plasmid DNA by this method was inefficient samples were prepared in quadruplicate and the DNA was pooled at a later step.

A cell pellet was obtained as described in section 2.3.5a and resuspended in 100 µl of 10 mM EDTA, 50 mM glucose in 25 mM Tris-HCl buffer, pH 8 and incubated on ice for 30 min with 2 mg/ml lysozyme. Bacterial lysis was completed by adding 200 µl of 1% SDS in 0.2 N sodium hydroxide and mixing gently. To precipitate chromosomal DNA, 150 µl of 3 M sodium acetate was added and the samples were placed on ice for 90 min. The chromosomal DNA and cell debris were removed by centrifugation for 10 min and the supernatant was transferred to a fresh Eppendorf tube. Any remaining precipitate was removed by emulsifying the supernatant in 0.5 ml phenol/chloroform and centrifuging the sample for 10 min. The

supernatant was removed to a clean Eppendorf tube. DNA was precipitated by the addition of 1 ml of absolute ethanol and held at -20 °C for 30 min. The DNA was pelleted by centrifugation for 5 min and the ethanol was discarded. The precipitate was dissolved in 100 µl of 100 mM/ 50 mM Tris-HCl and samples were pooled. Two volumes of 95% ethanol were added and the precipitation was repeated at -20 °C for 10 min. The plasmid DNA was pelleted by centrifugation for 2 min and the ethanol discarded. DNA was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, pH 8 containing 1 mM EDTA) and electrophoresed as described previously.

Gels from both methods were stained in 300 mls of ethidium bromide solution (1 µg/ml) for 30 min. Gels were examined under ultraviolet light and photographed.

2.3.6 Assays for adhesins

The presence of cell-associated factors, such as pili and haemagglutinins, which have been implicated in adherence, was investigated using tissue culture cell assays, haemagglutination, PCR (for *tcpA* genes) and electron microscopy (EM).

2.3.6a Adhesion to HEp-2 or Caco2 cells

Adhesion tests were performed using HEp-2 cell and Caco2 cell tissue culture systems. The method was based on that of Cravioto *et al.* (Cravioto *et al.* 1979). In some tests 1% D-mannose, which prevents attachment in *E. coli* strains to the tissue culture cells or other surfaces due to type 1 pili, was added. Maintenance of the tissue culture cells was as described in section 2.3.1. The HEp-2 cells were maintained in Basal Medium Eagle with Hank's salts and the Caco2 cells were maintained in Dulbecco's medium (see appendix 1). Two days prior to the test a monolayer of the appropriate cells was resuspended after trypsin treatment in growth medium without antibiotics. Sterile plastic tissue cluster plates containing a single sterile round coverslip per well, were seeded with 1.5 mls of tissue culture suspension

and incubated at 37 °C for 18 - 36 h until a monolayer of cells had grown.

The bacterial strains to be tested were grown overnight at 37 °C in peptone water, with or without mannose (1%). Prior to the test the tissue culture monolayers were washed with Earles Balanced Salt Solution (EBSS) and 0.25 ml bacterial culture were added to each well. For routine tests the monolayers were incubated for 3 h at 37 °C and washed 3 times with EBSS. The monolayers were fixed with methanol and then stained with 10% (v/v) Giemsa stain. The monolayers on the glass coverslips were cleared by dipping into acetone, acetone-xylene and xylene solutions, mounted onto glass slides using depex mountant and examined under oil immersion with a light microscope at 1000 x magnification. A minimum of 100 tissue culture cells was examined for each test. A test was considered positive when at least 40% of the tissue culture cells had 10 or more bacteria. Any tests with intermediate results were repeated, and the subsequent result was usually clearly positive or negative.

For certain tests the incubation was stopped at shorter times; for other tests the tissue culture medium was replaced at 3 h and incubation was continued for a further 3 h.

2.3.6b Haemagglutination tests.

The strains were grown overnight at 37 °C on slopes of CFA agar (Evans *et al.* 1977) or in peptone water. For the haemagglutination test the bacterial growth or bacterial pellet (from peptone water) was suspended in 0.15 M saline to give a concentration of 2×10^9 bacteria / ml. Fresh human, guinea-pig and rat blood were obtained by veno-puncture and cardiac puncture, respectively, and suspended in 0.4% (w/v) solution of sodium citrate in distilled water. Fresh bovine, horse and sheep whole blood cells in Alsevers solution were obtained from Tissue Culture Service. The blood was washed 3 times with PBS pH 7.4, and resuspended in PBS to make a 50% (v/v) stock suspension. This was used to make a 3% (v/v) suspension in saline. To assess the effect of mannose on haemagglutination, 0.5% (w/v) D-mannose was added to some tests. Using a pasteur pipette, one drop of bacterial suspension

and one drop of the 3% rbc suspension were mixed in a depression on a white porcelain tile. The tile was rocked by hand for 2 min. If no haemagglutination was seen the tile was placed on ice and rocked intermittently for a further 10 min. Haemagglutination was seen as a coarse clumping of the erythrocytes within one minute, but some strains gave only a fine granularity after 20 min. When haemagglutination took place with the red blood cell suspension which did not contain mannose, but not in that which did contain mannose, this reaction was recorded as mannose-sensitive (MSHA). Agglutination of rbcs suspended in saline containing mannose was called mannose-resistant (MRHA) (Duguid *et al.* 1979).

2.3.6c Detection of the gene for the toxin coregulated pilus (*tcpA*)

The gene for TCP (*tcpA*) was detected by a polymerase chain reaction described in section 2.3.3.

2.3.6d Electron microscopy

EM was used to examine strains for the presence of flagella and adhesive structures (pili). Strains for examination by EM were grown on CFA or nutrient agar at 37 °C for 24 h and subcultured for another 24 h incubation period. A thick suspension of the culture (c. 10^{10} cells/ml) was made in peptone water containing 4% formaldehyde. The suspension was diluted 1:2 in distilled water and 1 drop was applied to a Forval-carbon coated EM grid (400 mesh) for 3 min. Excess fluid was removed by blotting with filter paper, and a drop of 1% phosphotungstic acid, pH 6.4, was placed on the grid for 1 min before excess fluid was blotted as before. The grids were examined by transmission EM.

2.3.7 LPS analysis

A sensitive silver stain method was used for detecting lipopolysaccharide in SDS-polyacrylamide gels. (Tsai and Frasch 1982).

Bacteria were grown overnight on blood agar plates. The sample was prepared by weighing out approximately 10 mg of bacteria in a microcentrifuge tube. The sample was adjusted to 1 mg per 30 µl of solubilisation buffer (reagents are described in the appendix) and boiled at 100 °C for 15 min. An equal volume of proteinase-K in solubilisation buffer (1 mg/ml) was added and the sample was placed at 60 °C for 1 h. The proteinase-K digested the outer membrane proteins, leaving the LPS intact. 30 µl of digested material (equivalent to approximately 500 µg cells) was run on an SDS-PAGE gel. After electrophoresis (50 mAmp, 3.25 h), gels were fixed in 40% methanol/ 10% acetic acid, overnight. The next day the gel was placed in 0.7% periodic acid in fix solution (5 min). Then the gel was washed three times (15 min each) with distilled water. Silver stain was freshly prepared: 2 mls concentrated ammonium solution was added to 28 mls 0.1 M NaOH, then 5 mls 20% silver nitrate solution was added while stirring, the stain was made up to 150 mls with distilled water. The gel was stained for 10 min with vigorous agitation, then washed three times (10 min each) with distilled water. The colour was developed by reacting in 200 mls developer (50 mg citric acid and 1 ml 37% formaldehyde in 1 l water). The colour took at least 5 min to appear and the reaction was stopped by placing the gel in 10% acetic acid. The gels were photographed as soon as possible before discolouration occurred.

2.3.8 Iron chelating compounds (Siderophores)

Tris-succinate medium, as described by Braun (Braun 1981), was used to detect production of siderophores. This medium forces bacteria to use succinate as the major energy source, causing an apparent iron shortage due to excessive use of the cytochrome system. Also, this medium has the advantage of being colourless and does not interfere with subsequent colour reactions used for detecting siderophores.

2.3.8a Enterochelin

Enterochelin or enterobactin comprises catechol. The method of Arnow (Arnow 1937), used for the detection of catechols, can be used for the detection of enterobactin. Bacteria were grown in Tris-succinate medium at 37 °C with vigorous shaking. When cultures became turbid, bacteria in 2 mls of culture were sedimented (12,500 *g*, 10 mins). To 1 ml of supernatant was added 1 ml 0.5 M HCl, 1 ml nitrite/ molybdate reagent and 1 ml 1 M NaOH. A positive reaction gave a dark pink colour, the OD could be read at 505 nm using tris-succinate medium as a blank. In practice the test was easy to read by eye.

2.3.8b Aerobactin

The ferric perchlorate test for the detection of aerobactin was performed as previously described (Atkin *et al.* 1970; Payne 1980). The bacteria were grown in tris-succinate medium, as described above, and 1 ml of 5 mM ferric perchlorate in 0.14 M perchloric acid was added to 1 ml of supernatant. Aerobactin reacted to give a reddish colour, the test was read visually and at OD_{480}.

Both the aerobactin and enterochelin tests were successfully performed in microtitre (using 50 µl amounts). This had the advantage of allowing many strains to be tested and using less of the reagents. The microtitre tests were read visually.

2.3.9 Protein analysis

Whole cell and outer membrane protein analyses were carried out using variations of the methods of Laemmli (1970) as described by Chart and Griffiths (1985) (Laemmli 1970; Chart and Griffiths 1985).

2.3.9a Whole cell protein analysis

0.5 mls of an overnight culture was subcultured into 10 mls nutrient broth and

incubated at 37 °C for 4 h with shaking. The cells were harvested by centrifugation (3,000 *g*, 10 min) in a Sorvall 6000 centrifuge and the cell pellet was resuspended in 1 ml of PBS and transferred to a screw-cap Eppendorf tube. The cells were pelleted by centrifugation (12,500 *g*, 10 min) in an Eppendorf microcentrifuge and resuspended in 1 ml of distilled water. The cells were again centrifuged in an Eppendorf microcentrifuge for 2 min and 0.5 ml of lysis buffer (8% distilled water, 2% mercaptoethanol, 20% glycerol, 0.8 g SDS, 70% 0.5 M Tris/ HCl, pH 6.8) were added and mixed gently. The lysate was heated at 95 °C for 5 min in a Techne Dri-Block DB-1 (Jencons Scientific). 0.5 ml of distilled water was added and the tubes were returned to the block for a further 5 min. The lysate was centrifuged for 5 mins in an Eppendorf microcentrifuge and 0.8-1 ml of supernatant was transferred to a clean screw-cap Eppendorf tube. 10 µl of bromophenol blue (5 mls lysis buffer, 5 mls distilled water, 10 µg bromophenol blue) was added and samples were stored at -20 °C.

2.3.9b Outer membrane protein profiles

150 mls of an overnight broth culture were pelleted in a MSE centrifuge (6 x 250 ml rotor) at 5000 *g* for 30 min. The supernatant was discarded and the pellet resuspended in 5 mls of ice-cold Tris-HCl, pH 7.4 containing 1 mM EDTA. The suspension was placed in sonicator vials pre-cooled on ice and sonicated at 150 Watts for 3 min (Heat Systems, Ultrasonics, Inc) until a clear solution was obtained indicating cell lysis. The sonicated preparation was poured into a 50 mls centrifuge tube containing 20 mls ice-cold 25 mM Tris-HCl, pH 7.4 and unbroken bacteria were sedimented by centrifugation at 5000 *g* for 20 min in an MSE HS16 using the 8 x 50 ml rotor. Supernatants were poured into clean centrifuge tubes and cell envelopes were pelleted by centrifugation at 40,000 *g* for 1 h in an MSE HS16 at 4 °C using the 8 x 50 ml rotor. Outer membranes were separated from inner membranes by digesting the inner membranes with sodium lauryl sarcosinate (Sarkosyl). The envelope pellet was suspended in 20 mls of Tris-HCl, pH 7.4 and the protein content estimated

by measuring the OD_{280} (1 unit of OD_{280} approximately equals 1 mg protein ml preparation). For every 1 mg of protein, 20 µl of Sarkosyl were added and the tubes were incubated at room temperature for 30 min with vigorous shaking. Outer membranes were sedimented by centrifugation at 40,000 *g* for 1 h at 4 °C and the pellet was resuspended in 20 mls Tris-HCl, pH 7.4. Outer membranes were pelleted as before and the pellet was resuspended in 100 µl of Tris-HCL, pH 7.4, and stored at -20 °C.

2.3.9c Determination of protein using the Lowry procedure

Protein concentration was estimated using the method of Lowry *et al.* (Lowry *et al.* 1951). 20 µl of outer membrane protein preparations were added to 0.98 ml of distilled water. A protein standard of bovine serum albumin was also diluted in distilled water to give concentrations of 50, 100, 150 and 200 µg protein/ml. 5 mls of Lowry solution C (50 mls solution A + 1 ml solution B; see Appendix 1) were added to each tube, mixed thoroughly and left for 10 min. 0.5 ml of solution D (1 N Folin-Ciocalteau reagent) was added to each tube, mixed and left for 30 min. The optical density was read at 500 nm. OD_{500} was plotted against µg protein and the concentration of protein in each sample was calculated.

2.3.9d Electrophoresis of protein samples

30 µg of protein sample were run on a SDS-PAGE gel. The resolving gel was made in Tris-HCl, pH 8.6 with 12.5% acrylamide and the stacking gel in Tris-HCl, pH 6.8 with 5% acrylamide. The gel was run at 50 mA constant current per gel; the starting voltage was between 80-100 V and the final voltage 310 V. Gels were stained in a mixture containing 0.1% Page Blue 83 (BDH) in 25% solvent methanol, 10% glacial acetic acid and 65% distilled water. Before use, the stain was filtered through a 32 cm Whatman filter paper and the gels were allowed to stain overnight. Gels were destained by washing in several changes of staining mixture excluding the Page Blue 83.

RESULTS

3.0 Strains of *V. cholerae* and *V. mimicus* used in this study.

A total of 1323 strains of *V. cholerae* non-O1 and *V. mimicus* was used for this study, and which had been referred to the Laboratory of Enteric Pathogens (LEP). This group of organisms comprised:- 1177 strains of *V. cholerae* serogroup non-O1 and *V. mimicus* and 146 reference or control strains. These had been isolated from a range of sources and from 69 different countries (for more detail, please refer to Appendix 2). One hundred and thirty- nine strains were the reference strains for the serogrouping scheme of Sakazaki and Donovan (Sakazaki and Donovan 1984; Shimada *et al.* 1994) and the remaining 7 strains were control strains (WBDV-101E and NAG-ST) and five CT+ Australian strains described in section 2.1.

For comparison a total of 88 strains of *V. cholerae* belonging to serogroup O1 was also screened for CT and ST in this study; two strains (O1 Ogawa and O1 Inaba) were reference strains for the serogrouping scheme and one (E51116) was a CT+ O1 control, the remaining 85 were *V. cholerae* O1 referred to the LEP between 1985 and 1994.

The environmental isolates of *V. cholerae* non-O1 and *V. mimicus*, which account for 39% of strains, were from seafood (64%), water (31%) and sewage (4%). The remaining 1% of environmental strains were isolated from miscellaneous sources, including; gull liver, chicken feed and fish tanks. The seafood sources comprised a variety of fish and shellfish, however prawns and shrimps accounted for 86% of the total. The isolates from water were from both "fresh" water sources (27%), such as rivers and ponds, and from "salt"/ sea water sources (73%).

The human isolates of *V. cholerae* non-O1 and *V. mimicus*, 61%, were isolated

from the faeces of patients with diarrhoea, who had returned from abroad. Of these, more than half (52%), had visited one of four countries: these were India (116 cases), Kenya (115), Tunisia (80) or Thailand (64).

Fourteen of the human isolates were non-faecal isolates from extraintestinal disease, all these were *V. cholerae* non-O1 (Table 7). Strains of *V. cholerae* belonging to serogroup O1 and *V. mimicus* strains were not isolated from extraintestinal sites.

3.1 Typing of strains of *V. cholerae* and *V. mimicus*

The panel of strains of *V. cholerae* and *V. mimicus* which formed the basis for this study was typed by standard methods prior to use for further investigation.

3.1.1 Biochemistry

All strains of *V. cholerae* and *V. mimicus* were differentiated from other members of the genus *Vibrio* by salt (NaCl) requirement and the ability of strains to produce arginine dihydrolase. Strains of *V. cholerae* and *V. mimicus* are non-halophilic and arginine dihydrolase negative.

Strains of *V. cholerae* and *V. mimicus* produced oxidase. Strains of *V. cholerae* and *V. mimicus* always produced acid (shown by pink coloration in the alkaline peptone water sugars containing Andrades indicator) in glucose, mannitol, maltose and trehalose. The following biochemical tests were also positive for these organisms; Christensen's citrate (bright pink coloration in the slope), indole (dark pink ring observed after the addition of Kovac's reagent), lysine decarboxylase (purple colour), gelatin (due to the production of gelatinase the solid medium was liquefied) and nitrate (the bright red colour observed after the addition of indicator reagents A and B showed the reduction of nitrate to nitrite).

Table 7: Strains of *V. cholerae* belonging to serogroup non-O1, isolated from extra-intestinal locations

Ref.no.	serogroup	location*	country§	Patient details		
				dia†	age‡	sex
E43504	O?	appendix	Saudi Arabia	?	?	?
E39404	O?	bile	Iran	N	84	M
E39381	O5	blood	?	?	75	F
E43854	O11	blood	Arab Emirates	?	?	?
E48238	O8	blood	Jordan	?	?	?
E55130	O42	blood	India	?	?	?
E87187	O-rough	blood	Caribbean	?	?	?
E87739	O?	blood	Saudi Arabia	?	29	F
E94383	O-rough	blood	?	?	78	M
E68192	O51	ear	?	?	19	M
E73275	O?	ear	?	?	22	F
E55414-6	O?	?wound	?	?	46	M
E91926	O?	wound	?	?	?	M
E100095	O?	blood	North Africa	?	?	M

* Site of isolation

§ Country visited prior to infection

† Presence or absence of diarrhoea

‡ Patient's age in years

Strains of *V. cholerae* and *V. mimicus* did not produce gas from glucose (observed in a Durhams tube), and did not utilize the following sugars by fermentation (the Andrades indicator remained pale yellow); raffinose, salicin, dulcitol, inositol, adonitol, sorbitol, arabinose, rhamnose, xylose, inulin, sorbose and melibiose. *V. cholerae* and *V. mimicus* were also negative in the following biochemical tests; mucate (the test remained blue), urea (no pink coloration was observed), arginine dihydrolase (yellow colour), H_2S production (no blackening of the glucose iron agar slope was observed), KCN (these organisms did not grow in potassium cyanide), malonate and PPA (no colour changes observed in medium or after addition of indicator reagents for PPA reaction).

V. cholerae and *V. mimicus* could be distinguished from each other by VP (Vogues-Proskauer), sucrose fermentation and ONPG (O-nitrophenyl-ß-galactosidase) (Table 8).

3.1.2 Biotyping

Strains of *V. cholerae* belonging to serogroup O1 can be differentiated into two biotypes, classical and El Tor by: haemolysis of horse red blood cells (rbc), chick rbc haemagglutination, VP test, PB 50 sensitivity (polymixin B discs at 50 Units per disc obtained from Oxoid) and sensitivity to phage 3 (classical phage) and phage 4 (El Tor phage) of the USSR *V. cholerae* phage typing scheme.

Strains of *V. cholerae* serogroup non-O1, and *V. mimicus* did not react with either the classical or El Tor phages. With respect to haemolysin production on horse rbc (section 3.6) and polymixin sensitivity most *V. cholerae* non-O1, including O139, and *V. mimicus* were "El Tor-like" (Table 9). The VP reaction was variable for strains of *V. cholerae* non-O1, but all strains of *V. mimicus* were negative with the VP test. The ability of strains of *V. cholerae* non-O1 and *V. mimicus* to haemagglutinate chick erythrocytes was not tested.

Table 8: Biochemical differentiation of *V. cholerae* and *V. mimicus*

	V. cholerae	*V. mimicus*
NaCl* requiring	-	-
production of lysine decarboxylase	+	+
production of ornithine decarboxylase	+	+
production of arginine dihydrolase	-	-
indole	+	+
VP	v§	-
ONPG	+	+(94%)
utilization of sucrose	+	-
utilization of arabinose	-	-
PB 50†	v	+(95% resistant)

* can grow in the absence of NaCl, § reaction variable, † polymixin B discs

Strains of *V. cholerae* belonging to classical and El Tor biotypes, did not agglutinate chick red blood cells.

3.1.3 Serotyping

All test strains of *V. cholerae* could be identified by serology using the scheme of Sakazaki and Donovan (Sakazaki and Donovan 1984). The scheme was also applied to strains of *V. mimicus* but not to the halophilic vibrios. The serogrouping scheme was used with 1177 strains of *V. cholerae* non-O1 and *V. mimicus*, and the results are depicted in Figure 4. Of these strains, 343 (30%) were shown to belong to 59 distinct serogroups; 663 (56%) strains were unidentifiable (O?) with antisera raised against serogroups O1 (Ogawa and Inaba), O2 through to O83, O139 and a presumptive serogroup 5609 (see 3.11.2) and the remainder (171 (14%)) were considered rough. As these results are only based on the limited antisera available for evaluation (belonging to serogroups from O2 through O83 and O139) the typability of the scheme may be improved by the recently expanded scheme described by Shimada *et al* (Shimada *et al.* 1994).

Fifty-five strains of *V. mimicus* were also examined with this serogrouping scheme. Thirty-five (64%) strains were unidentifiable, none were rough and 20 (36%) were agglutinated by test sera. These strains reacted with sera prepared to serogroups O6 (2 strains), O14 (3), O20 (3), O34 (1), O39 (3), O41 (1), O43 (4), O71 (1) and 5609 (2), the number of strains reacting with the given serum is shown in parentheses. None of the represented serogroups was exclusive to *V. mimicus*.

It appeared that certain serogroups were associated with strains isolated from either the environment or from patients. Strains of *V. cholerae* belonging to serogroup O19 were most frequently isolated from environmental sources, whereas strains belonging to serogroups O2, O5, O9, O11, O13, O24, O34, O37 and O139 were isolated predominantly from cases of diarrhoea (Table 10). Seventy percent (238/343) of the strains which could be

Table 9 : Comparison of biotyping of strains of *V. cholerae* belonging to serogroups O1, O139, non-O1 and *V. mimicus*.

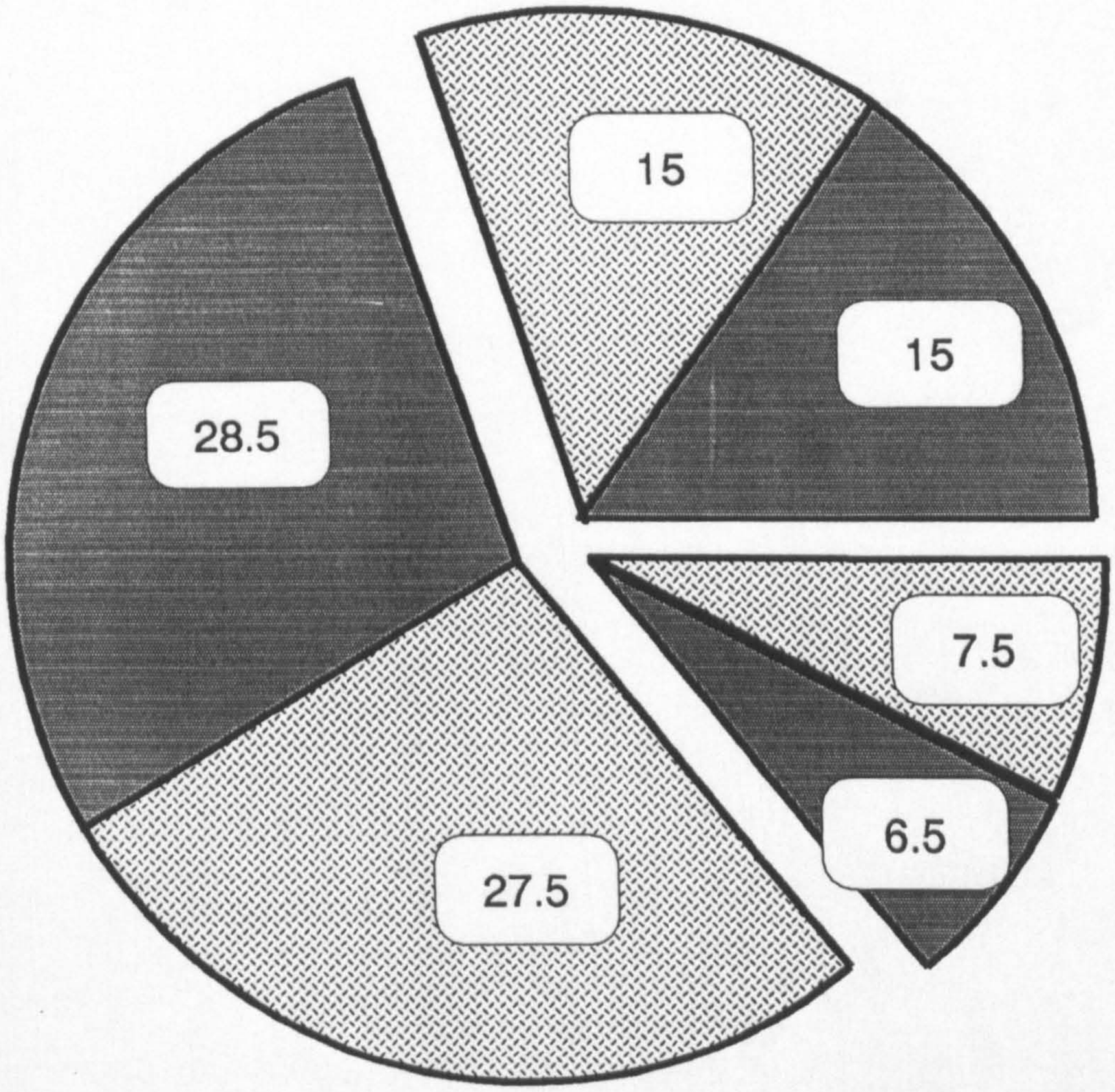
	V. cholerae				*V. mimicus*
	O1 classical	O1 El Tor	O139	non-O1	
Haemolysin*	-	+	+	+(90%)	+(90%)
VP	-	+	+	v	-
PB 50†	S	R	R	v	R
Phage 3	+	-	-	-	-
Phage 4	-	+	-	-	-

* production of haemolysin as detected with horse red blood cells

† polymixin B discs

v = variable; S = sensitive; R = resistant

Figure 4: Serogrouping of *V. cholerae* non-O1 and *V. mimicus* using the scheme of Sakazaki & Donovan (1984), incorporating antisera to serogroups O2 to O83 and O139.



	Human	Environmental	
Typable	30%	15%	15%
Unidentifiable	56%	28.5%	27.5%
Rough	14%	6.5%	7.5%

strains isolated fom clinical sources

strains isolated from environmental sources

identified by this serogrouping scheme belonged to the 20 serogroups (one of which was presumptive serogroup, 5609, identified in this study, 3.11.2) listed in Table 10.

Strains of *V. cholerae* belonging to serogroup O2 (25 strains, 21 of which were isolated from patients with diarrhoea) and O9 (24 strains, 17 of which were isolated from patients with diarrhoea) were most commonly encountered, and both serogroups have been responsible for family outbreaks (Table 11). A *V. cholerae* O2 caused diarrhoea in a mother and her two young children who had visited Egypt and a *V. cholerae* O9 caused diarrhoea in a mother and daughter who had visited Tunisia. None of these strains possessed the genes for either CT or ST.

All the strains listed in Table 11 were implicated in family outbreaks, however, serogrouping and R-typing demonstrated that the isolates from three of these "outbreaks" were not the same. Of the three strains from Malta, although only 2 had an identical R-type, all 3 strains were indistinguishable by serogrouping (all O?) and identical by LPS analysis (3.11.1).

3.1.4 Phage-typing

Strains of *V. cholerae* were also typed using the USSR phage typing scheme as described by G. Bertram, M.Phil. (Bertram 1990; Drozhevkina and Artuyunov 1979). Seven phages were used; phages 1, 2, 3 and 7 were Mukerjee phages for the typing of the classical biotype and phages 4, 5 and 6 were specific for the El Tor biotype. The phages were used at the dilution which gave semi-confluent lysis on an indicator strain, this dilution is the routine test dilution. A phage reaction ranging from greater than 20 plaques to confluent lysis was considered positive. The pattern of lysis given by the 7 phages determined the phage type; phage types 1 to 6,17 and 19 were associated with the classical biotype of serogroup O1, while phage types 10 to 16 and 18 were associated with the El Tor biotype (Table 12). If the strain reacted with the phages but did not give a known pattern it was recorded as non-conforming (NC). If there was no reaction with the phages the strain was considered untypable (U).

Table 10: The most common serogroups of *V. cholerae* non-O1 and association with source.

Serogroup	No. isolated from patients	No. isolated from environment	Total (%)
O2	21	4	25
O3	4	3	7
O5	11	1	12
O6	4	4	8
O9	17	7	24
O11	8	1	9
O13	10	2	12
O14	4	7	11
O19	1	12	13
O24	11	0	11
O34	10	3	13
O37	9	3	12
O39	5	6	11
O41	3	4	7
O49	2	4	6
O58	3	9	12
O76	4	4	8
O79	4	5	9
O139	13	0	13
5609	7	8	15
			Total =238 (70%)
Other serogroups	59	46	105 (30%)

Table 11: Strains of *V. cholerae* associated with family outbreaks.

Ref. N°.	Serogroup	R-type*	Country visited	patients' details age (years)	sex
E66824	O?	not tested	Tunisia	41	Male
E66825	O39	not tested	Tunisia	26	Female
E70987	O-rough	Ampicillin	Kenya	43	Male
E70988	O?	Colomycin	Kenya	26	Female
E71329	O2	Colomycin	Egypt	1	Male
E71330	O2	Colomycin	Egypt	4	Female
E71331	O2	Colomycin	Egypt	38	Female
E73155	O?	Colomycin, Ciprofloxacillin	Malta	46	Female
E73156	O?	Ampicillin, Colomycin	Malta	48	Male
E73160	O?	Colomycin, Ciprofloxacillin	Malta	?	Female
E73467	O49	Colomycin, Ciprofloxacillin	India	23	Female
E73468	O?	Colomycin, Trimethoprim, Ciprofloxacillin	India	25	Female
E98995	O9	Colomycin	Tunisia	55	Female
E98996	O9	Colomycin	Tunisia	28	Female

* Resistance type

Table 12: Phage typing patterns for USSR scheme.

Lysis by typing phage							Phage type
1	2	3	4	5	6	7	
+	+	+	-	-	-	+	1
-	+	+	-	-	-	+	2
+	-	+	-	-	-	+	3
-	-	+	-	-	-	+	4
+	+	+	-	-	-	-	5
+	+	+	-	-	+	+	6
+	-	-	+	-	+	-	10
-	-	-	+	+	-	+	11
-	-	+	+	-	-	-	12
-	-	-	+	+	+	+	13
-	-	-	+	-	+	-	14
-	-	-	+	-	-	-	15
-	-	-	-	-	+	-	16
-	-	+	-	-	-	-	17
+	-	+	+	-	-	-	18
-	-	-	-	-	-	+	19

Table 13: Phage typing results with strains of *V. cholerae* non-O1.

Ref. N°.	serogroup	phagetype (1)	phagetype (2)
CA385	O-rough	3	3
B4202-64	O5	NC	6?
7007-62	O6	19	U
10317-62	O8	NC	NC
112-68	O9	NC	NC
218-68	O10	NC	NC
317-71	O23	19	U
12795-62	O30	19	U
171-68	O32	NC	U
151-68	O33	NC	NC
1322-69	O37	NC	NC
112-73	O44	2?	17
122-73	O45	19	U
128-73	O46	19	NC
133-73	O48	19	NC
1463-76	O57	19	U
113-79	O73	17	U
1421-77	O80	19	U
E38780	O26	19	U
E38820	O2	15	U
E39056	O?	15	U
E39719	O-rough	NC	U
E39974	O22	15	14
E40016	O?	15	U
E40387	O?	19	U
E40454	O?	19	U
E41307	O?	NC	Not Done
E42751	O41	NC	U
E42869	O?	NC	Not Done
E42994	O?	NC	U
E43640	O2	19	U
E43867	O?	19	U
E45312	O41	NC	U
E46846	O76	14 or 16	14
E46989	O?	phage 10 +	U
E48238	O8	phage 11 +	phage 11+
E54918	O-rough	phage 11 +	U
E54381	O37	phage 11 +	U
E54048	O?	phage 11 +	U
E55879	O?	NC	NC

The first 18 strains are reference strains and all E numbers are wild type strains.
NC = non-conforming.
U = untypable.

A panel of reference strains comprising strains belonging to serogroups O2 through to O83, O-rough and O139 were phage-typed (Table 13). Of these 84 reference strains, 18 (21%) initially reacted with the test phages and of these 11 strains gave results correlating with established phage lysis patterns. The O-rough reference strain (which was originally derived from a serogroup O1, classical biotype) was the only strain to give a consistent phage type (PT 3).

The first 500 wild-type strains of *V. cholerae* non-O1 (see Appendix 2) were also phage-typed; 22 (4.4%) initially reacted with the panel of phages, and of these only 10 gave phage reactions which conformed to known patterns (Table 13). A further 5 strains were positive with "new" phages described by G. Bertram (Bertram 1990); phage 10 was a variant of the USSR phage 5 and phage 11 was a carried phage isolated from a serogroup O1 biotype El Tor strain from Hong Kong.

The reproducibility of phage results was examined by repeating the phage typing procedure. Results in Table 13, column designated "phage-type (2)", show that the results of phage typing were not consistent and therefore the scheme was not considered reproducible for strains of *V. cholerae* belonging to serogroups other than O1.

3.1.5 Resistance (R)-typing

A total of 300 strains of *V. cholerae* non-O1, isolated during the 2 year period 1992-94, was tested for resistance to ampicillin (A), chloramphenicol (C), colomycin (Co), ciprofloxacillin (Cx), furazolidone (Fu), gentamicin (G), kanamycin (K), streptomycin (S), sulphthiazole (Su), spectinomycin (Sp), tetracycline (T) and trimethoprim (Tm).

Of these 300 strains, 8% (25/300) were sensitive to all 12 antibiotics tested, the remaining strains showed varying degrees of resistance. The most common resistance pattern observed, in 56% (169/300) of strains, was resistance to colomycin only. A further 24% (72/300) of strains showed resistance to colomycin along with resistance to other antibiotics.

The only other single antibiotic resistance pattern observed was for ampicillin, and 3% (10/300) of strains were resistant to this antibiotic alone. Ampicillin resistance along with resistance to other antibiotics was observed in 10% (31/300) of strains. The commonly observed resistance patterns and the percentage of strains resistant to each antibiotic tested are shown in Table 14.

There appeared to be no correlation of R-type with serogroup, except for strains of *V. cholerae* belonging to serogroup O139. Strains of *V. cholerae* O139 were consistently found to be resistant to CoSSuTm; three strains including the type strain were also resistant to Fu and one strain was also resistant to Sp and T (ie: CoSSuSpTTm). The CoSSuTm resistance pattern was distinctive and closely associated with the O139 serogroup, however, the same resistance pattern was also found in four other strains (E80120 (O?), E90655 (O38), E90645 (O?), and E97773 (O?)) all isolated from patients who had recently returned from India.

CT-positive strains belonging to other serogroups other than O139 were not found to be multiply resistant to antibiotics (section 3.3).

3.1.5a Resistance to O/129 and polymixin.

In general, strains of *V. cholerae* and *V. mimicus* were sensitive to the vibriostatic agent O/129; however, approximately 15% of strains were resistant. Resistance to O/129 was closely associated with resistance to trimethoprim, and all strains of *V. cholerae* belonging to serogroup O139 were resistant to O/129.

Polymixin sensitivity of *V. cholerae* and *V. mimicus* was described in section 3.1.2.

Table 14: Resistance of V. cholerae non-O1 to antibacterial drugs and commonly observed resistance patterns.

Antibiotic	% of V. cholerae non-O1 resistant
A = ampicillin	13%
C = chloramphenicol	2%
Co = colomycin	80%
Cx = ciprofloxacillin	8%
Fu = furazolidone	9%
G = gentamicin	0
K = kanamycin	0
S = streptomycin	10%
Su = sulphthiazole	13%
Sp = spectinomycin	3%
T = tetracycline	1%
Tm = trimethoprim	5%

Resistance pattern	number of strains (%)
Co	169 (56%)
sensitive	25 (8%)
CoSSuTm	14 (5%)
A	10 (3%)
ACo	6 (2%)
AFu	6 (2%)
CoSu	6 (2%)
CoCx	5 (2%)
other	59 (20%)

3.2 Detection of toxins with cultured cells.

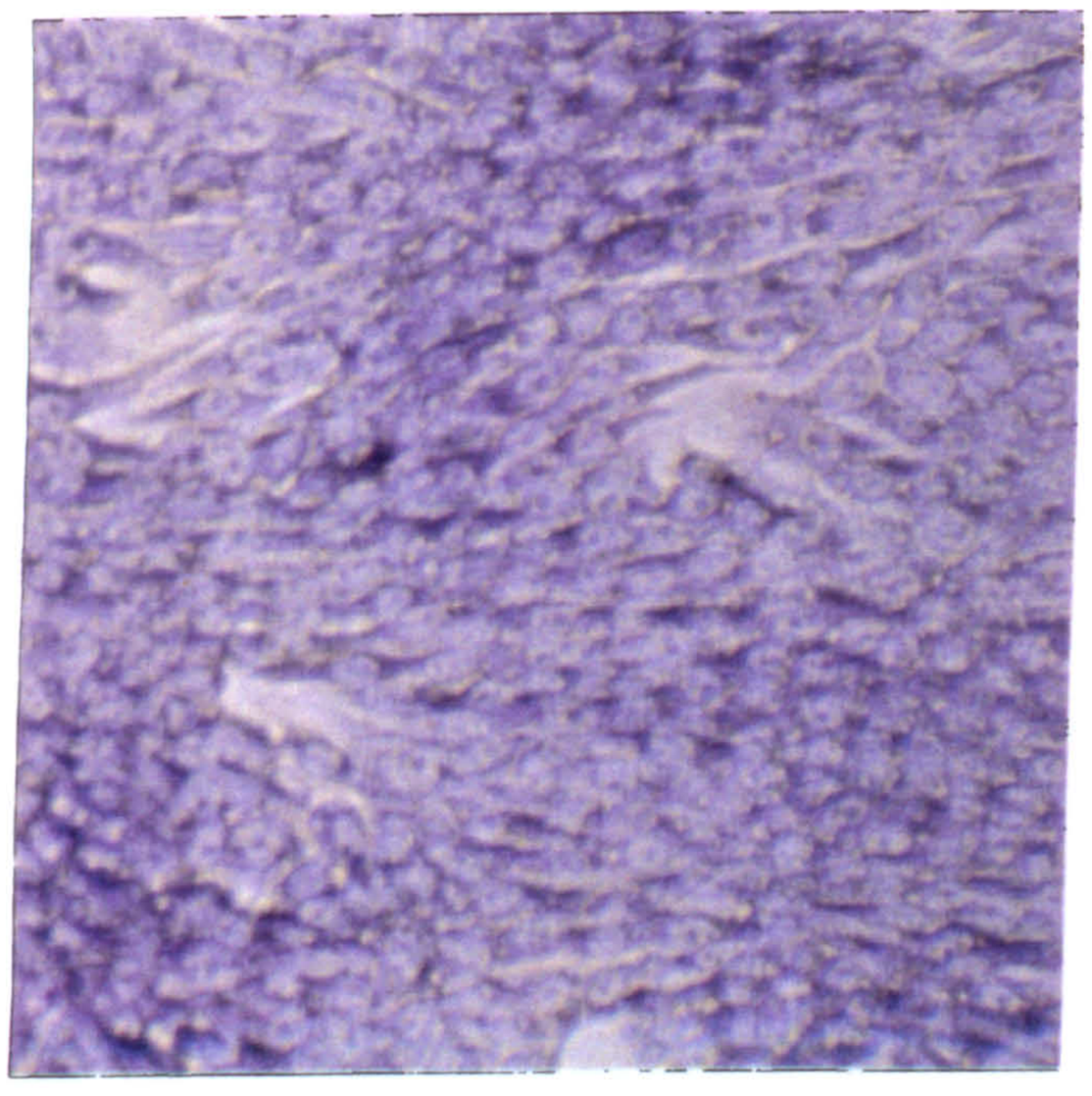
3.2.1 Morphological effects of toxins, produced by *V. cholerae* non-O1, on Y1, HeLa and Vero cell-lines.

Cell tests were performed using culture filtrates as described (Section 2.3.1a). Reference strains of *V. cholerae* belonging to serogroups O2 to O40, and 200 wild type strains (5 *V. mimicus* strains and 195 *V. cholerae* non-O1 strains) were used for these experiments. The LT+ *E. coli* B7A, CT+ *V. cholerae* O49 (WBDV-101E) and three *V. cholerae* O1 (E51116 CT+, 10954/1 Ogawa CT+, 8457/5 Inaba CT-) were also included.

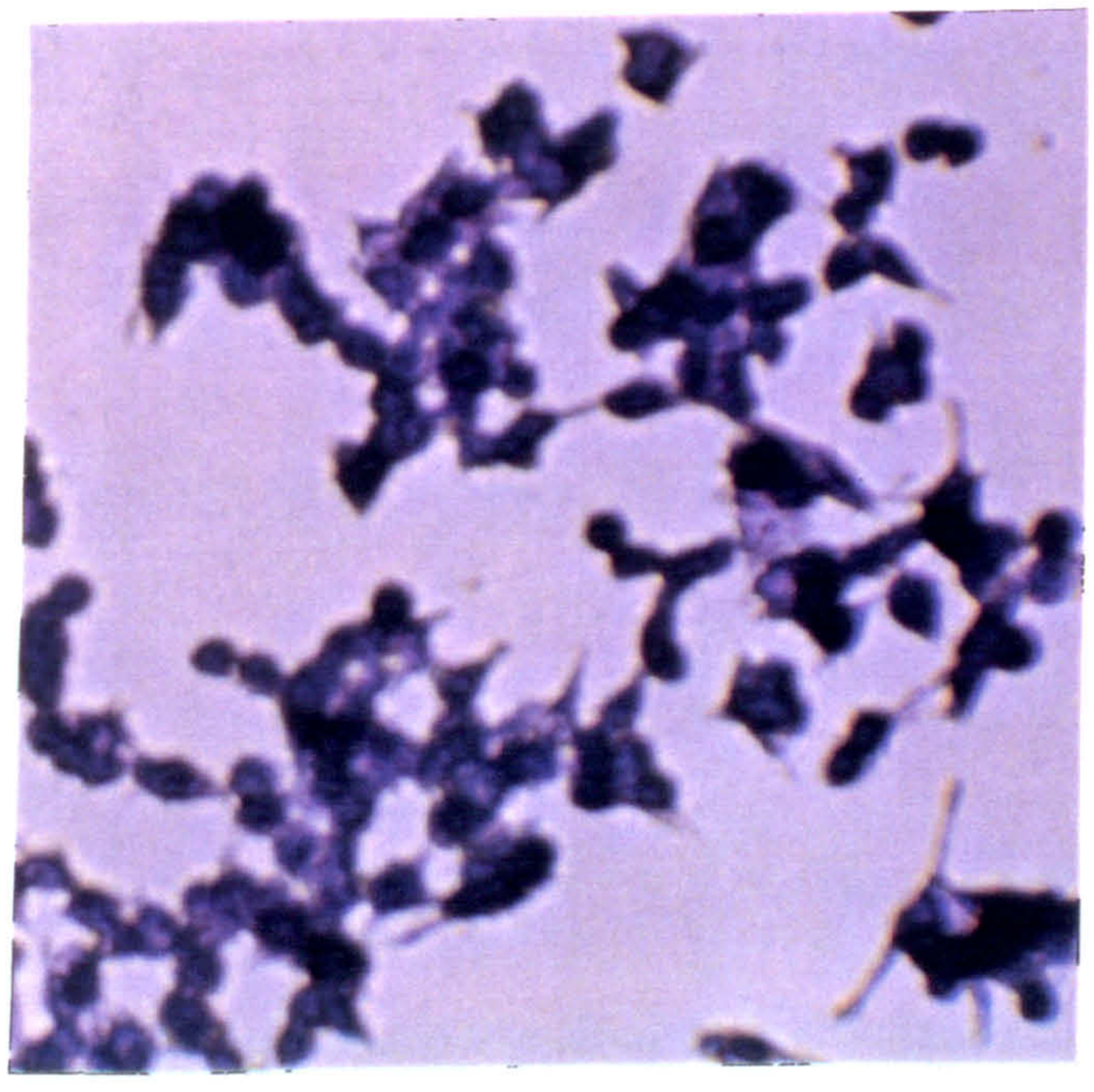
The protocol described for detecting *E. coli* LT (Section 2.3.1b) formed the basis for detecting toxins produced by strains of *V. cholerae*. Crude preparations of toxin were prepared from broths following growth of strains in TSB. Preparations were added to Y1, HeLa and Vero cells, and plates stained with Geimsa's stain and examined after 1 day and 3 days. The effect of the heated preparation was compared with that of the unheated to ensure that the observed effects of the unheated preparation on the tissue culture cells was due to a heat-labile factor. All the effects, described below, were heat-labile. A test was scored as negative if the cells remained intact at the end of the test.

CT and *E. coli* LT characteristically lead to rounding of the Y1 cells. In the presence of CT or LT the test was very clear with over 90% of cells rounded (Figure 5). This rounding effect was a cytotonic response which was reversed if the Y1 cell test was extended to 3 days. *E. coli* B7A, *V. cholerae* O-rough (reference strain CA385) and *V. cholerae* O1 (reference strain 10954/1) produced the typical cell rounding effect on Y1 cells. However, the CT-negative *V. cholerae* O1 (reference strain 8457/5) and the CT-positive *V. cholerae* non-O1 strains (reference strain 1322-69 (O37) and control strain WBDV-101E (O49)) were cytotoxic.

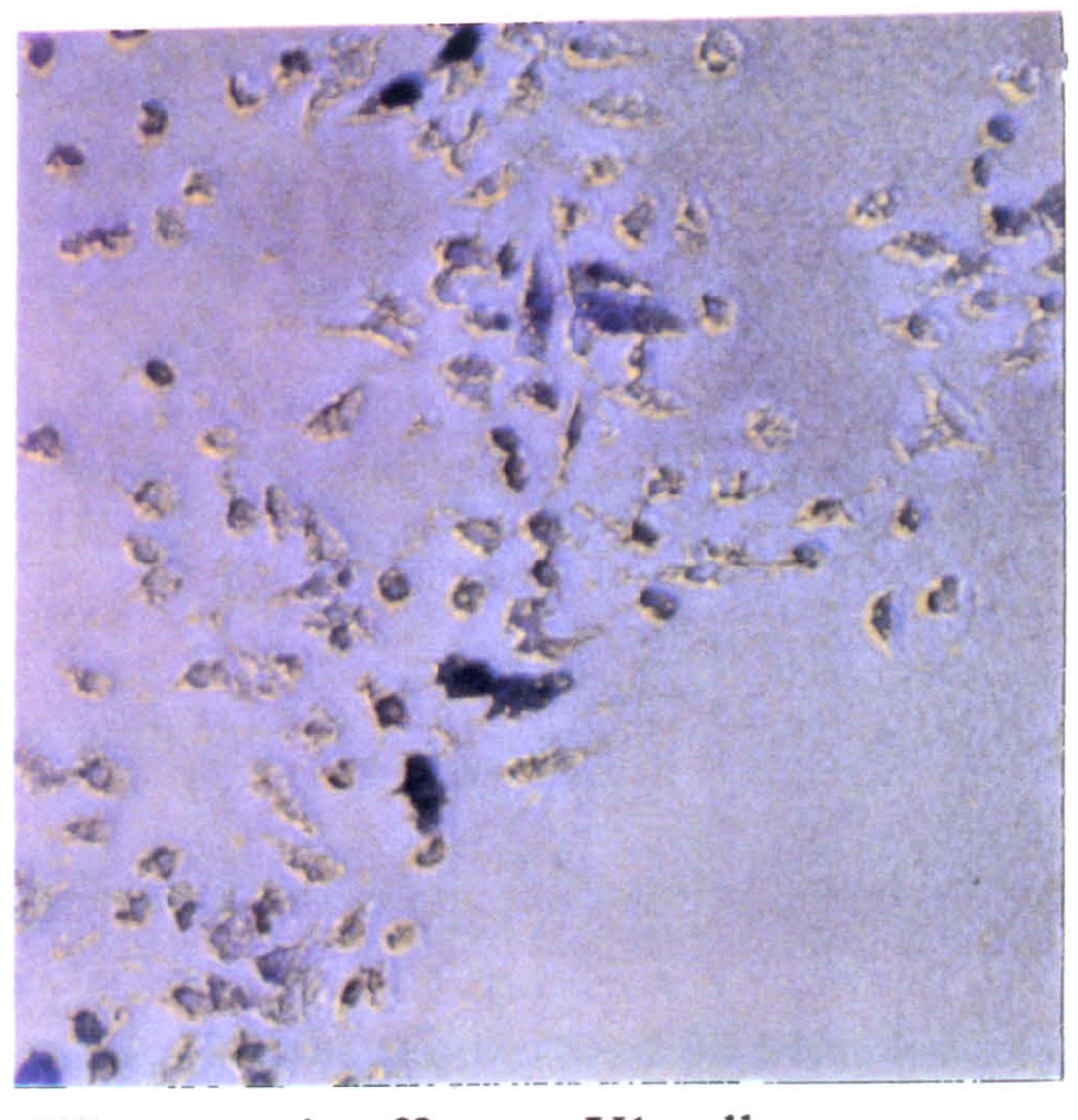
Figure 5: Morphological effects on the Y1 cell line.



(i) Normal Y1 cell monolayer



(ii) rounding (cytotonic) effect on Y1 cells

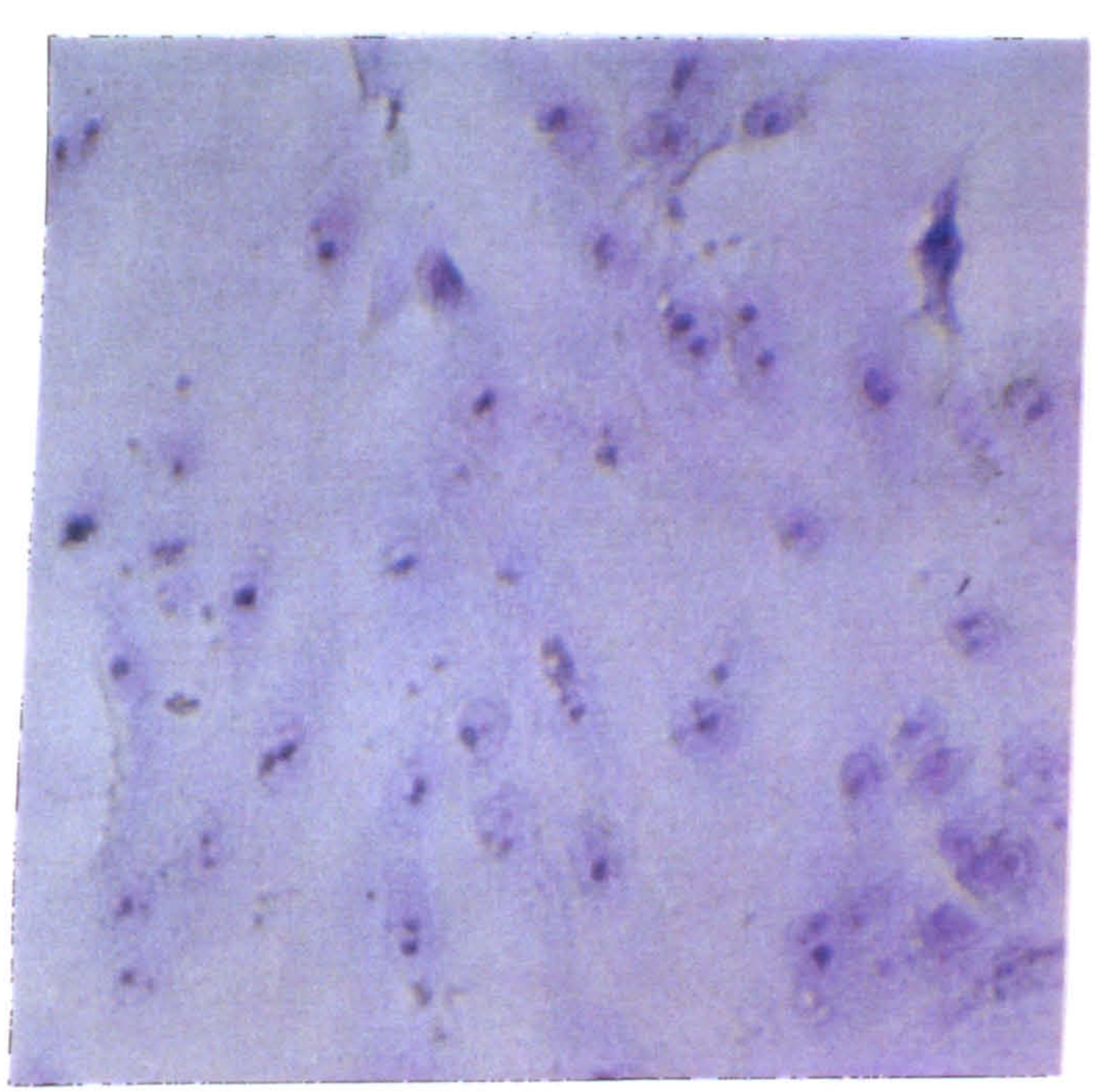


(iii) cytotoxic effect on Y1 cells

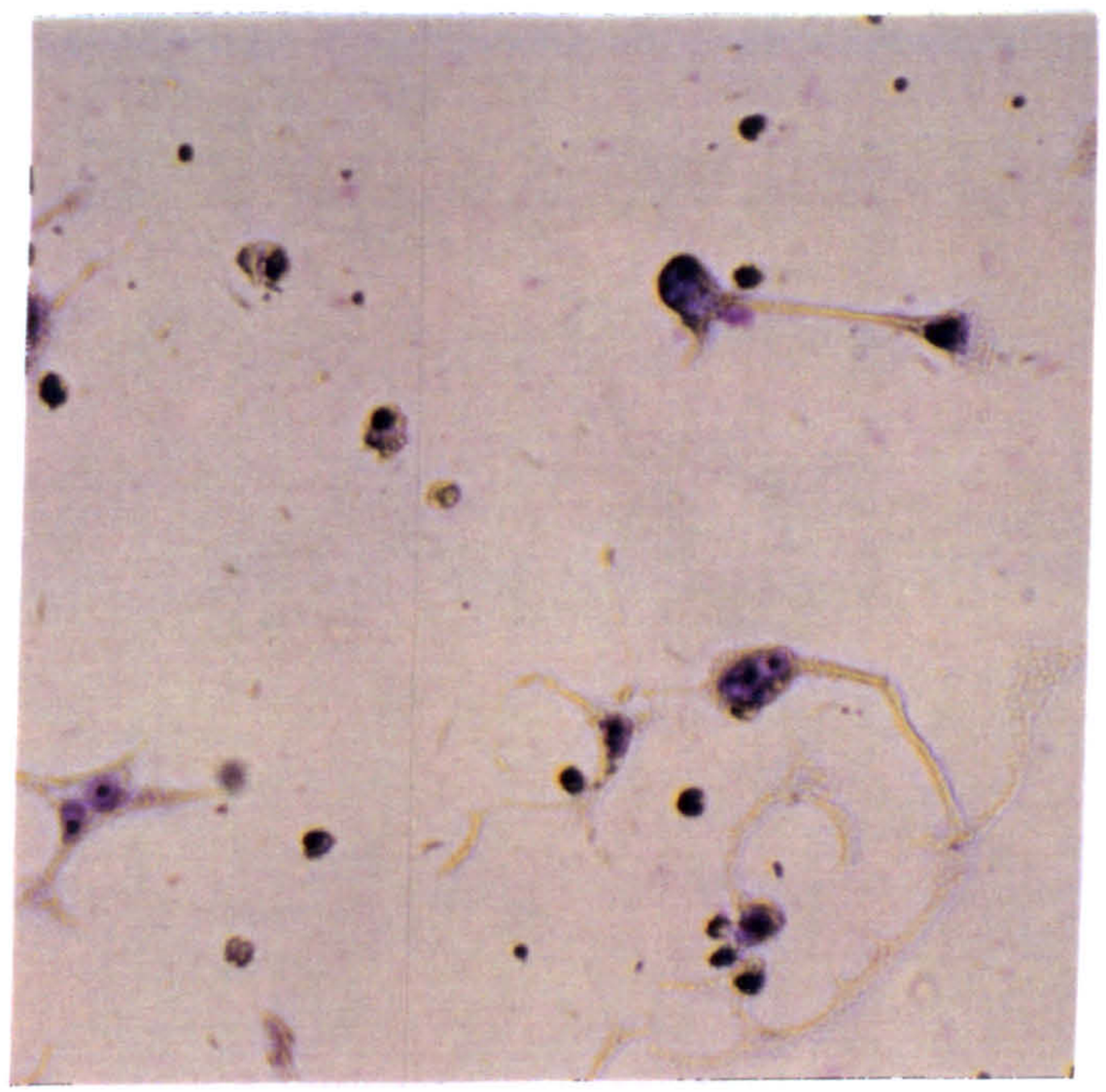
The observed effects with 88% of culture filtrates of CT-negative *V. cholerae* non-O1 were also cytotoxic (irreversible cell damage, leading to cell death) varying from slight elongation of cells to complete cell destruction with plate wells becoming devoid of cells (Figure 5). It was not possible to tell if the different morphological appearances on Y1 cells (elongation, "ghosting" and "shrivelling") were different stages of a single cytotoxic effect or if the effects were distinct from each other, therefore all these effects were recorded as cytotoxin (c). In the presence of cytotoxin(s) between 80% and 100% of Y1 cells were found to be affected. Both the rounding effect of LT and CT and the cytotoxic effects were seen after 1 day in Y1 cells. True rounding was confirmed as CT or LT by neutralisation tests (2.3.1b) using antisera raised against these toxins, cytotoxic effects were not neutralised by the antisera. Three strains of *V. cholerae* non-O1 (E60391, E60393, both O? isolated from shrimps, and E42878, an O13 isolated from a patient who had been travelling in India) produced a "rounding" effect which could not be neutralised by antisera to LT or CT and was irreversible, that is cytotoxic rather than cytotonic. These 3 strains also produced cytotoxic effects on Vero and HeLa cells, they were therefore regarded as cytotoxin producers. The Vero cell line was also sensitive to LT and CT, in the presence of these toxins the cells rounded up and become partially detached from each other, however this effect was only seen in a 1 day test, by 3 days the monolayer had recovered from these cytotonic toxins. In the presence of cytotoxin the entire Vero cell monolayer became detached and few cells were left (Figure 6). In the absence of cytotoxin the monolayer remained intact. As well as the cytotoxic effect another distinct irreversible heat-labile effect was the vacuolation (v) of Vero cells (Figure 6).

The Vero cell line is usually used to detect the Vero cytotoxin (VT) of *E. coli* (Konowalchuk *et al.* 1977; Scotland *et al.* 1980). VT is part of a group of toxins which are closely related to the Shiga toxin of *Shigella dysenteriae* type 1 the causative organism of dysentery, therefore VT is also known as Shiga-like toxin (SLT). VTs are cytotoxic for Vero cells and these toxins cause death of Vero cell monolayers. Neutralisation tests using antisera

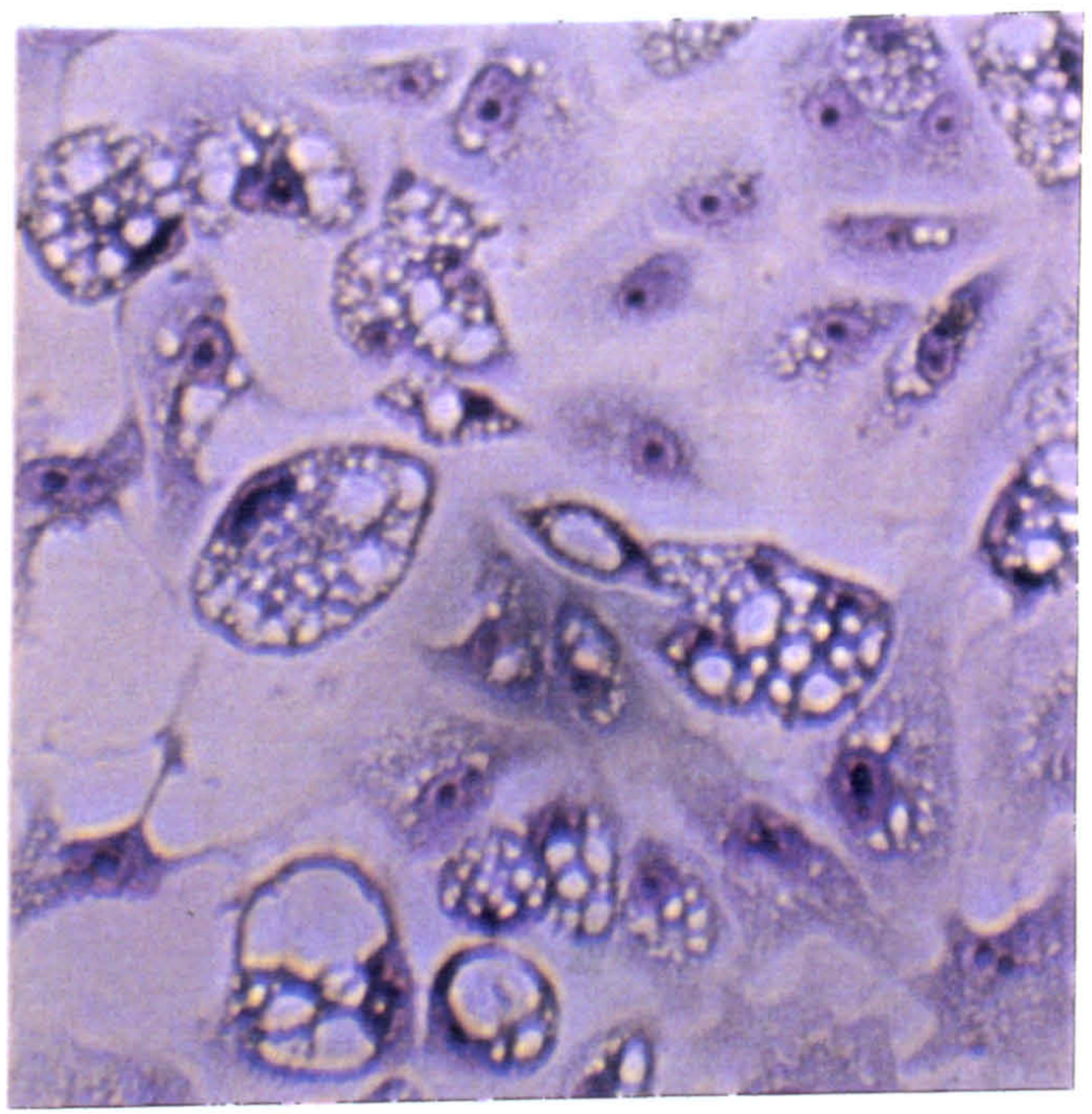
Figure 6: Morphological effects on the Vero cell line.



(i) Normal Vero cell monolayer



(ii) cytotoxic effect on Vero cells



(iii) vacuolation on Vero cells

prepared against VT (VT1 and VT2) did not neutralise the cytotoxic effect produced by *V. cholerae* non-O1 culture filtrates on Vero cells. It was concluded that the cytotoxic effect observed was probably not due to the activity of VT or VT-like toxins (see also VT probe, 3.5.2).

The HeLa cell line was the most resistant to morphological effects produced by the culture filtrate preparations. The cells of the HeLa monolayer have a rounded appearance which made it difficult to distinguish rounding of the HeLa cells in response to LT or CT. In the presence of cytotoxin more than 50% of the cells became detached, the remaining cells often appeared to have lost their characteristic round shape and had "protrusions" extending from the cell.

Using the three cell lines (Y1, Vero and HeLa) there were six observed patterns of cytotoxin production (Table 15). The most common pattern observed was cytotoxicity on all three cell lines (47%), only 12% were negative (no cytotoxin production detected on the 3 cell lines). The vacuolating toxin was produced on Vero cells by 16% of *V. cholerae* non-O1 strains, this was most commonly associated with a shrivelled appearance on Y1 cells, with no observed effect on HeLa cells. All five *V. mimicus* strains tested were cytotoxic on all three (ccc) cell lines. There was no apparent correlation of cytotoxic pattern with serogroup. Cytotoxin production was most frequently observed with strains of *V. cholerae* belonging to serogroups O2 (10 cytotoxic strains/10 strains tested), O5 (6/6) and O19 (6/9). Strains belonging to serogroups commonly isolated from patients with diarrhoea (O2, O5, O13 and O34) were cytotoxic on Y1 cells and belonged to patterns 1, 3, 4 and 5 (Table 15). Of the strains belonging to the O19 serogroup, commonly isolated from environmental sources, only 1 was cytotoxic on all three cell lines (pattern 1), 3 strains were negative and the remaining 5 strains belonged to pattern 2 (c-c).

Table 15: Morphological effects of toxins produced by strains of *V. cholerae* non-O1 on Y1, HeLa and Vero cells.

Pattern	No. of strains (%)	Vero	Hela	Y1
1	112 (47%)	c	c	c
2	21 (9%)	c	-	c
3	10 (4%)	-	c	c
4	29 (12%)	-	-	c
5	38 (16%)	v	-	c
6	29 (12%)	-	-	-

c = cytotoxin

v = vacuolating toxin

3.2.2 Toxin production in different growth media.

As described above (3.2.1) *E. coli* B7A and certain *V. cholerae* strains (O-rough CA385 and O1 10954/1) produced the typical cell rounding effect on Y1 cells when grown in TSB. However, the CT production of known CT-probe-positive (3.3.1) *V. cholerae* non-O1 (O37 1322-69 and O49 WBDV-101E) was not demonstrated, due to the production of cytotoxin, which masked CT production. The use of different culture media and different growth conditions were used to optimise conditions for the production of CT (this is described in more detail in section 3.3.3). Although the masking effect of the cytotoxin could not be overcome, it was found that *V. cholerae* non-O1 toxin titres were highest when grown in Syncase Sucrose broth at 37 °C with aeration.

3.3 Detection of cholera toxin (CT)

Due to the fact that certain strains produced cytotoxin(s), it was difficult to detect CT using tissue culture cell assays (3.2). To avoid this problem, specific DNA probes and immunoassays for CT were used.

3.3.1 DNA probes for CT

The possession of CT genes, by strains of *V. cholerae* non-O1 and *V. mimicus* was investigated using polynucleotide probes CTA and CTB, described in 2.3.2b, SNAP-LT (a commercial kit) described in 2.3.2f, and an oligonucleotide probe GM1, described in 2.3.2g.

The first probe used was an ^{35}S labelled polynucleotide probe to CTB, prepared by Dr. G. Willshaw and described by G. Bertram, M.Phil (Bertram 1990). It was found that approximately 80% of the ^{35}S was incorporated into the probe, which could be used for up to 3 months. For colony hybridisation broth cultures of test strains were "spotted" onto nylon

membranes, as described (2.3.2d). The radio-labelled CTB probe was hybridised with nylon membranes at 42 °C and 37 °C to test type strains (O-rough, O2 - O83) at both "high" and "low" stringency. Reference strains of *V. cholerae* O-rough and O37 were positive with this probe at both temperatures. Forty four strains of *V. cholerae* non-O1, isolated from humans and 45 strains isolated from environmental sources, were also tested using high and low stringency temperatures. All 89 strains were found not to react with the gene probe.

With the aim of finding a safer alternative to using radio-labelled probes, the use of enzyme-labelled probes was investigated. A commercially available synthetic nucleic acid (SNAP) probe designed to detect the LT of *E. coli* was tested. This SNAP-LT kit was compared with the ^{35}S-labelled CTB probe for the detection of CT, in addition to the detection of LT gene sequences. With the SNAP-LT kit only LT-positive *E. coli* tested positive, all 172 strains of *V. cholerae* tested were negative, including known CT-positive strains of *V. cholerae* belonging to serogroups O1, O37 and O-rough. In contrast the radio-labelled probe detected both LT and CT positive strains. It was concluded that the SNAP-LT probe was inappropriate for testing strains of *V. cholerae*.

It was decided to enzyme-label the polynucleotide CT probes which were available. The first enzyme-labelling system used was a biotin label with the polynucleotide CTA probe, as described by G. Bertram, M.Phil (Bertram 1990). However, it was found that the colony blot results were difficult to interpret and chromosomal blots gave false positive results. An alternative enzyme-labelling method, using digoxigenin (2.3.2c), was tried and was found to detect CTA and CTB genes sequences using colony blots. The digoxigenin labelling system was also successful with oligonucleotide probes, such as GM1, therefore this labelling procedure was used for subsequent studies.

Table 16: Incidence of CT genes in wild type strains of *V. cholerae* and *V. mimicus*

	Total no. tested	CTA	CTB	CT+ %
V. cholerae O1	85	74	74	87%
V. cholerae non-O1	1122	15	15	1%
V. mimicus	55	0	0	0%

3.3.1a Digoxigenin-labelled CTA and CTB

Strains of *V. cholerae*, belonging to serogroups O1 (85 strains) and non-O1 (1122 strains), and *V. mimicus* (55 strains) were tested for the possession of genes encoding CT using digoxigenin-labelled CTA and CTB polynucleotide probes (Table 16). Initially, strains were tested in parallel with probes for both CTA and CTB; however, since the strains either reacted with both or did not react with either probe,a mixed CTA+B probe (equal volumes of CTA and CTB) which gave strong positive results (Figure 7) was used for further screening. When CT-positive strains were detected, these were tested with the separate CTA and CTB probes to ensure genes for both toxin subunits were present. All strains detected with the mixed CTA and CTB probes were found to have the genes for both CTA and CTB.

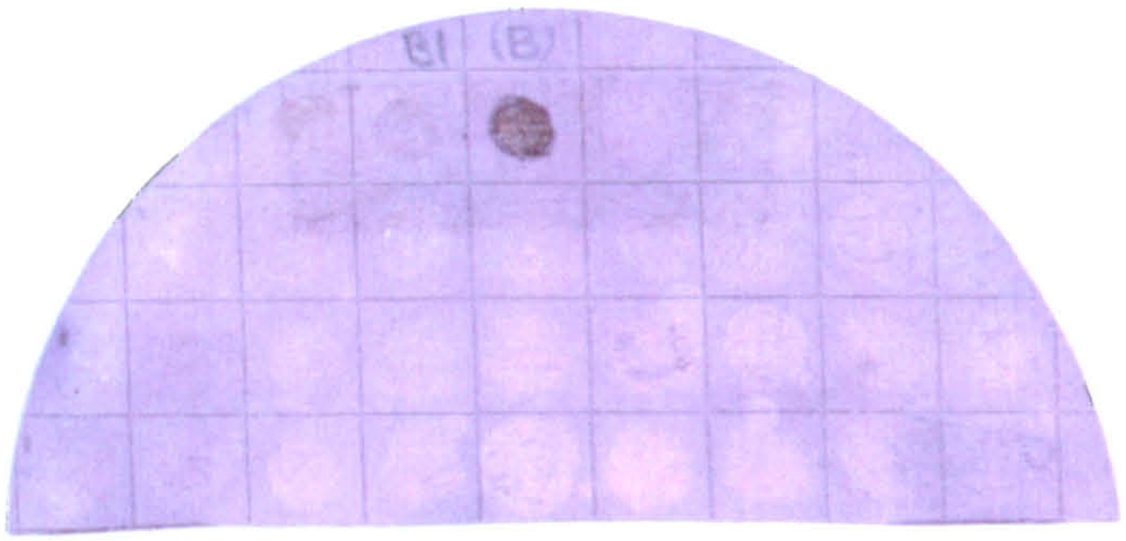
The combined CTA+B probe was also used at "low" stringency, which allowed both LT and CT gene sequences to be detected (Figure 7). Any strains of *V. cholerae* non-O1 or *V. mimicus* possessing gene sequences related to CT and LT should be detected at low stringency. Strains which were positive in the GM1-ELISA (see Section 3.3.2) but CT-probe-negative at high stringency were suspected of producing a CT-like toxin, however, 119 GM1-ELISA positive strains were tested at low stringency with the CTA+B probe and all were negative. The number of strains reacting with the DNA probes was not increased when the stringency was lowered. None of the *V. mimicus* strains tested and only 1% of *V. cholerae* non-O1 (13 of these belonged to serogroup O139) tested were CT-positive (a full list of CT-positive strains is given in Table 17).

The 141 reference strains belonging to serogroups O1 Inaba, O1 Ogawa, O-rough and O2 to O139 (the non-O1 reference strains comprise 131 *V. cholerae*, and 9 *V. mimicus*) were also examined for reaction with CTA+B probes. Of 141 strains 6 were positive (Table 17), 5 positive strains were *V. cholerae* and 1 strain was a *V. mimicus*. The possession of the genes for CT was found to correlate with the somatic antigens expressed by test strains.

Figure 7: Digoxigenin-labelled CTA and CTB probes

Hybridisation of combined CTA+B gene probe at 68 °C.

The positive colony blot is *V. cholerae* O37 (1322-69), the remaining colony blots are CT-negative *V. cholerae* non-O1 and an LT-positive *E. coli* B7A.



Hybridisation of combined CTA+B gene probe at 37 °C showing LT-positive *E. coli*.

The positive colony blots are *V. cholerae* strains O1 (E51116), O37 (1322-69), O? (E55879), O? (E66371) and O49 (WBDV-101E) and an LT-positive *E. coli* B7A, the remaining colony blots are CT-negative *V. cholerae* non-O1.

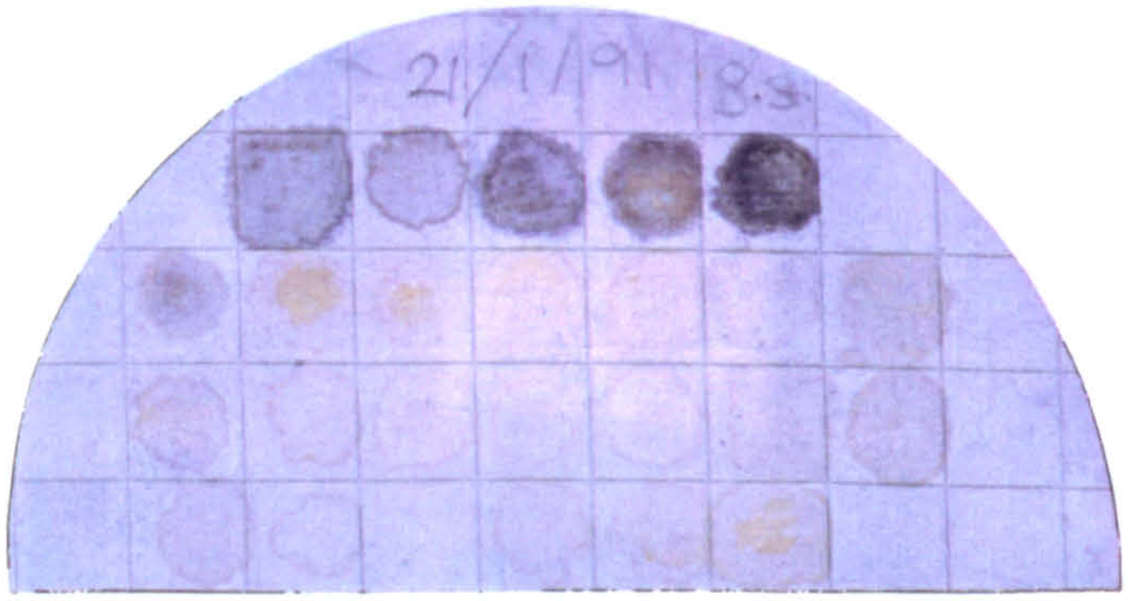


Table 17: List of CT gene positive strains of *V. cholerae* non-O1 and *V. mimicus*

(Including reference strains and control strains).

Reference No.	Serogroup	Country of origin	Patient details diarrhoea/age/sex* or source of strain
CA385	O-rough	?	reference strain
10954/1	O1 Ogawa	?	reference strain
1322-69	O37	?	reference strain
571-88	O105	?	reference strain
523-80 (*V. mimicus*)	O115	?	reference strain
MO45	O139	?	reference strain
E51116	O1 Ogawa	Bangladesh	control strain
WBDV-101E	O49	?	control strain
N2	O6	Australia	isolated from water
N87	O6	Australia	isolated from water
N7	O23	Australia	isolated from water
N9	O23	Australia	isolated from water
N92	O23	Australia	isolated from water
E85943	O139	?	D ? ?*
E86270	O139	Bangladesh	D 36 M
E87509	O139	India	D 68 ?
E87954	O139	India	D 25 F
E88131	O139	India	D 19 M
E89007	O139	India	D 30 M
E90067	O139	Pakistan	D ? ?
E90070	O139	Pakistan	D ? ?
E90171	O139	Pakistan	D ? ?
E90251	O139	Thailand	D 25 F
E92660	O139	Bangladesh	D 54 M
E99892	O139	Pakistan	D 33 F
E100069	O139	India	D 33 F
E55879	O?	Egypt	D 22 M
E66371	O?	Tunisia	D 46 M

* Patient details; ? = unknown, D = diarrhoea, age = stated in years, M = male, F = female

Of the strains of *V. cholerae* belonging to serogroup O1, 87% (74) were found to possess the genes encoding CT, and 100% of strains of *V. cholerae* belonging to serogroup O139 carried genes encoding CT. In contrast, <1% of *V. mimicus* (a single strain; 523-80 the reference strain for O115) and *V. cholerae* non-O1/non-O139 reacted with the probes.

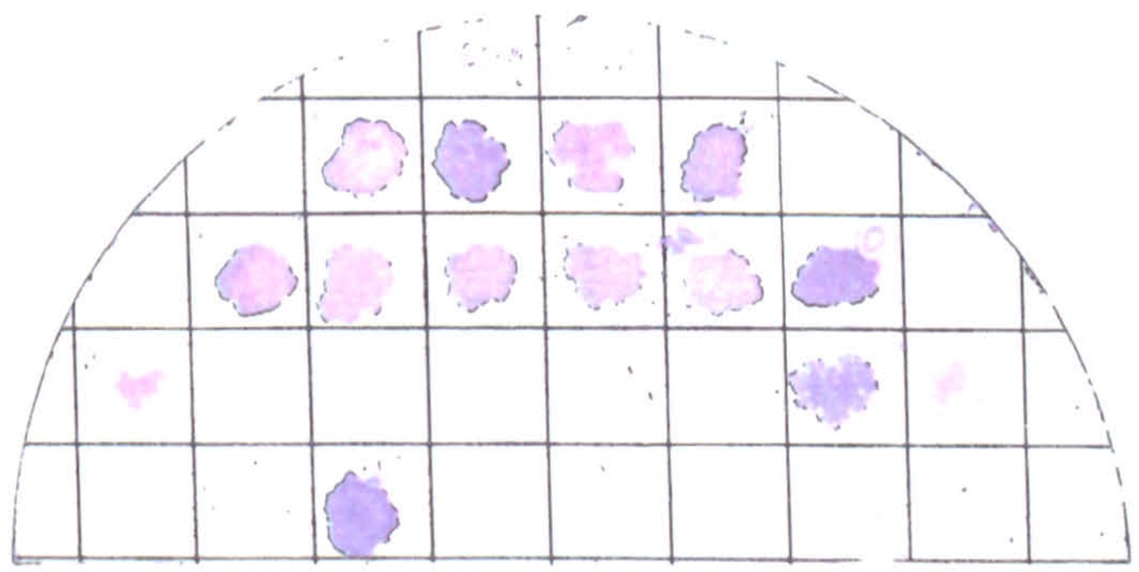
3.3.1b GM1 oligonucleotide probe

The B-subunits of CT bind to the ganglioside GM1, and the DNA sequence encoding this part of the CTB molecule has been determined. An oligonucleotide probe to the GM1 ganglioside binding part of the CT gene, was labelled with digoxigenin and used in hybridisation studies. The GM1 probe hybridised with strains of *E. coli* producing LT and CT-producing strains of *V. cholerae* at 48 °C (Figure 8). Of the 500 *V. cholerae* non-O1 (including the 119 CT probe-negative, GM1-ELISA positive strains (Section 3.3.2a), only the strains known to be CT-positive from previous hybridisation experiments were positive with this probe.

3.3.2 Immunoassays

As tissue culture cell assays could not be used to demonstrate CT production due to the masking effect of cytotoxins, immunoassays were used as an alternative means of demonstrating CT production by strains of *V. cholerae* and *V. mimicus*. Small scale filtrates (described in section 2.4.2a) were used to test large numbers of strains in these immunoassays. ELISA and VET-RPLA assays confirmed the CT-positive strains. All CT probe negative strains tested were also negative in the VET-RPLA. However, in the GM1-ELISA some CT-negative strains gave reproducible positive results and these strains were investigated further using probe tests (see 3.3.1a and 3.3.1b above) and other immunoassays, such as PIH, Biken assay and colony immunoblotting in an attempt to confirm the production of a CT related heat-labile toxin.

Figure 8: Hybridisation with digoxigenin-labelled GM1 oligonucleotide probe at 48 °C



The positive colony blots are *V. cholerae* strains belonging to serogroups O1 (E51116), O37 (1322-69), O49 (WBDV-101E), O139 (MO45, E85943, E86270, E87509, E87954, E88131, E89007), O-rough (CA385) and LT-positive *E. coli* B7A. The remaining strains are CT-negative *V. cholerae* non-O1.

3.3.2a GM1-ELISA

The GM1-ELISA uses the ganglioside GM1 to coat ELISA plates and "capture" toxin in sterile culture filtrates. The probe positive *V. cholerae* non-O1 strains, the *V. cholerae* O1 (Table 17) and *E. coli* strain B7A were tested in the GM1-ELISA using antisera raised against CT (LEP), CTB (Novabiochem) and LT (LEP). All CT-positive and LT-positive strains were positive in the GM1-ELISA test; positive culture filtrate consistently gave a reading of >1.0 (OD_{405}).

Culture supernatants, prepared from 630 probe-negative strains of *V. cholerae* non-O1 were also tested using the anti-CT, anti-CTB and anti-LT. Of the 630 strains, 119 (19%) gave a positive result with anti-CT and anti-CTB, but gave a negative result with an antiserum prepared to LT (LEP). It was concluded that this putative toxin had the ability to bind the ganglioside GM1 and shared antibody binding sites with CT, and more specifically with CTB.

Strains of *V. cholerae*, giving positive results with the GM1-ELISA, were reacted with the CTA+B combined probe at low stringency (3.3.1a) and with the GM1-oligonucleotide at 48 °C (3.3.1b), however all strains remained probe-negative.

3.3.2b VET-RPLA

The VET-RPLA was a reversed passive latex agglutination kit (2.3.1e) which was used to detect CT and LT. The 119 probe-negative, GM1-ELISA positive strains of *V. cholerae* (3.3.2a) were negative by latex agglutination. All CT-probe positive strains (listed in Table 17) and the *E. coli* LT-positive strain (B7A) were, as expected, positive by VET-RPLA.

The VET-RPLA was also used to assess the effect of different growth conditions on CT-probe-positive *V. cholerae* (3.3.3).

3.3.2c Double sandwich ELISA

Selected probe-negative, GM1-ELISA positive strains (which consistently gave OD_{405} of 1.8 - 2.0), were tested in another ELISA system where the antisera raised against LT (LEP), CT (LEP) and CTB (Novabiochem) were used as first ligand, replacing the GM1 ganglioside. An appropriate second ligand, dependent on the antiserum chosen as first ligand, was used (2.3.1d). Four different combinations were possible (Table 18), a positive filtrate gave a reading of >0.9 at OD_{405}.

CT-probe positive strains *V. cholerae* O49 (WBDV-101E), *V. cholerae* O1 (E51116), LT-positive *E. coli* B7A and 8 strains of probe-negative, GM1-ELISA positive *V. cholerae* non-O1 were used in these experiments. Syncase sucrose broth (SSB) was used as a negative control. The culture filtrates were prepared after growth in SSB and toxin production was also tested in the GM1-ELISA (using anti-CT), the VET-RPLA and the Y1 cell assay (Table 18). The most successful double sandwich ELISA used anti-LT as first ligand and anti-CTB as the second. The two ELISA tests in which anti-CTB was used as a first ligand were totally negative. The OD_{405} for the double sandwich ELISAs were lower than in the GM1-ELISA and three strains gave results which were indeterminate (OD_{405} >0.5 and <0.9).

3.3.2d Biken test

The Biken test detects the LT of *E. coli* by the formation of a precipitin line between test colonies and antisera raised against the toxin to be detected (eg: anti-LT). Three test strains of *V. cholerae* and a positive control strain *E. coli* B7A were included on each plate. The line of precipitation was observed in a light-box.

This test worked well with LT-positive *E. coli* B7A using either anti-LT (LEP), anti-CT (LEP) or anti-CTB (Novabiochem). Two CT-positive *V. cholerae* O1 (E51116 and 10954/1), 3 CT-positive *V. cholerae* non-O1 (1322-69, WBDV-101E and E55879), 10 probe-negative, GM1-ELISA-positive *V. cholerae* non-O1 and 2 probe-negative, GM1-ELISA-

Table 18: Comparison of Double Sandwich ELISA, GM1-ELISA, VET-RPLA and Y1 cell assay.

Strain	Double sandwich ELISA First ligand Second ligand	LT CTB	CT CTB	CTB LT	CTB CT	GM1-ELISA	VET-RPLA	Y1 cell assay[§]
E. coli B7A		+(0.932)[*]	+(0.889)	-(0.190)	-(0.258)	+(1.878)	+	+
V. cholerae O1		+(0.997)	+(0.816)	-(0.146)	?(0.598)	+(2.000)	+	+
V. cholerae O49		+(0.950)	+(0.812)	-(0.149)	-(0.435)	+(1.999)	+	c
E47180		?(0.546)	?(0.593)	-(0.168)	?(0.555)	+(1.964)	-	c
E47279		+(1.191)	+(1.009)	-(0.090)	?(0.608)	+(1.934)	-	c
E46827		+(0.922)	+(0.895)	-(0.116)	?(0.599)	+(1.991)	-	c
E54418		+(0.955)	+(0.909)	-(0.110)	-(0.222)	+(1.961)	-	c
E55414		?(0.777)	?(0.735)	-(0.097)	-(0.312)	+(1.949)	-	c
E58860		+(1.175)	+(1.265)	-(0.117)	-(0.245)	+(1.887)	-	c
E61246		+(1.099)	+(1.008)	-(0.105)	-(0.231)	+(1.824)	-	c
E62127		?(0.713)	?(0.725)	-(0.174)	?(0.501)	+(1.988)	-	c
SSB (negative control)		-(0.222)	-(0.219)	-(0.182)	-(0.212)	-(0.253)	-	-

[*] ELISA results are recorded as + = positive, - = negative and ? = indeterminate. The OD405 reading for the ELISA tests is given in parentheses.

[§] The Y1 cell assay is recorded as + = cytotonic toxin (typical rounding) and c = cytotoxic effect.

negative *V. cholerae* non-O1 strains were tested against the 3 antisera. All *V. cholerae* strains grew poorly on the Biken agar compared with the growth of the *E. coli* strain and all were negative with anti-LT and with anti-CTB. However, with anti-CT weak precipitin lines were observed with the CT-probe-positive strains, and some weaker precipitin lines were observed with two of the probe-negative, GM1-ELISA-positive strains (E47279 and E46827).

3.3.2e Passive immune haemolysis (PIH)

The PIH test (2.3.1f) was also originally developed for the detection of LT, however this test has been used to detect CT-like toxin of *V. cholerae* non-O1 (Yamamoto *et al.* 1981). Six strains of *V. cholerae* non-O1 (all GM1-ELISA positive), *V. cholerae* O1 (E51116), *V. cholerae* O37 (1322-69) and *E. coli* B7A were used. The LT and CT positive strains were tested in the tube assay to ensure the system would detect LT and CT, then all the strains were tested in microtitre.

Although Yamamoto *et al.* (Yamamoto *et al.* 1981) found they could use this system with their strains and the LT positive could be detected in both the tube and microtitre systems; neither the tube nor the microtitre PIH method was appropriate for the *V. cholerae* tested. This was because these strains produced haemolysin in their culture filtrates in addition to CT, therefore, the SRBC were lysed before the end of the antibody adsorption period (ie: before the test could be completed). The culture filtrates of the *V. cholerae* strains were also titrated in the microtitre system using doubling dilutions, the wells were examined for haemolytic activity before and then after the addition of complement. All the strains gave haemolytic titres of 1/20 or greater before and after addition of complement, one strain E55879 (O?, CT+) was the only strain which showed an increased haemolytic titre after the addition of complement, from 1/20 to 1/80, this increase may have been due to PIH induced by CT.

3.3.2f Colony and supernatant immunoblotting

The colony immunoblotting technique relies on the transfer of protein toxin, in this case CT or CT-related toxins, to Hybond-C extra nitrocellulose membranes, the toxin is detected using antisera and alkaline-phosphatase conjugated IgG. Colony immunoblotting was used together with antiserum raised against CT (LEP) and CTB (Novabiochem). Twenty-three *V. cholerae* non-O1 strains were grown on nutrient agar plates for 6 h, a nitrocellulose membrane was placed gently onto the colonies for 5 min, and the procedure carried out as described (section 2.3.1h). Seventeen strains were probe-negative, GM1-ELISA positive, 4 strains were positive for both CT probe and ELISA and 2 strains were negative in both tests. However, all 23 strains gave a positive reaction with both anti-CT and anti-CTB (a strong dark purple colour), within 5 min.

In order to remove cell debris which may be interfering with the colony immunoblotting, the same strains were grown in SSB and filtered supernatants prepared (2.1.3a). The supernatants were spotted directly onto the nylon membranes and the detection procedure carried out as for colony immunoblotting. However, the results were the same, all 23 were positive, indicating that the reaction may not be specific.

3.3.3 Optimising growth conditions for the production of CT.

The culture conditions used for growing strains of *V. cholerae* non-O1 influenced the result of the tissue culture tests. In particular the masking of CT production by cytotoxin in the Y1 cell assay was a problem (3.2.1). Therefore, with the aim of optimising culture conditions for CT production, the use of different culture media and different growth conditions was assessed. Strains of *V. cholerae* which possessed CT genes (CT-probe-positive) and produced CT as detected by immunoassays (VET-RPLA and ELISA positive), were used. Strains were as follows: reference strain *V. cholerae* O37 (1322-69), control strain *V. cholerae* O49 (WBDV-101E), *V.cholerae* non-O1 wild strain E55879 (O?), *V. cholerae* O1 (E51116) and

E. coli (B7A). Culture filtrates of these strains were prepared after growing the organisms in Syncase sucrose broth (SSB), Syncase glucose broth (SGB), Trypticase soy broth (TSB), Brain heart infusion broth (BHI) and Casamino yeast extract broth (CYE) (2.3.1a). The culture conditions, for each culture medium, were varied by temperature (30 °C or 37 °C) and by growing statically or with aeration. Doubling dilutions of filtrate were used to determine titres of heat-labile enterotoxin and cytotoxin. The titre is the reciprocal of the highest final dilution in each respective test causing toxic effect. Neutralisations with antiserum against CT or LT were performed to confirm specificity of the rounding. The effect of media and different growth conditions on toxin production was assayed using Y1 cells, neutralisation assays, VET-RPLA and GM1-ELISA (Table 19).

V. cholerae non-O1 strains produced cytotoxin(s) which made it impossible to detect the true rounding of CT on Y1 cells under any of the growth conditions used, even when filtrates were diluted to 1/156250, cytotoxin was still present. The cytotoxic effect present in the filtrates was destroyed by heating at 100 °C for 15 min (as was CT) but the effect was not neutralised by anti-cholera toxin. The control *V. cholerae* O1 (E51116) strain did not produce cytotoxin under these same conditions, but did cause typical rounding of the cells which was confirmed as CT by neutralisation. Similarly, *E. coli* strain B7A produced rounding typical of LT and this effect was neutralisable. For the *V. cholerae* O1 strain the highest CT titre (2560) was obtained at 30 °C in SSB with aeration. *E. coli* B7A gave the highest titre (156,250) at 37 °C with aeration, in either SSB or TSB.

The effect of growth conditions on CT production by the probe positive strains, could easily be detected and quantified in the immunoassays. As shown in Table 19, there were conditions under which no toxin production could be detected by either immunoassay (ELISA or VET-RPLA). The highest CT titres for *V. cholerae* non-O1 strains, as detected by VET-RPLA, were obtained with SSB at 37 °C, with aeration: these were titres of 32 for wild type strain E55879 (O?) and 64 for both reference strain 1322-69 (O37) and control strain

WBDV-101E (O49). Although the masking effect of the cytotoxin could not be overcome, it was concluded that *V. cholerae* non-O1 toxin titres were highest when grown in SSB at 37 °C with aeration.

3.3.3a Kinetics of production of CT and LT

Bacteria were grown in 20 ml volumes of SSB with aeration at 37 °C. At 2, 4, 6, 8, 12 and 24 hours post inoculation, 2 ml volumes were removed, bacteria sedimented and the supernatants filtered through 0.2 μm Millipore filters. The filtrates were assayed for CT or LT in GM1-ELISA, VET-RPLA and Y1 cell assay; the production of cytotoxin was detected on Y1 cells. The strains used were; *V. cholerae* O1 (E51116), *V. cholerae* O37 (1322-69) and *E. coli* B7A (Table 20 and Figure 9).

The ELISA and VET-RPLA tests confirmed the production of LT and CT, even in the filtrate of the O37 strain which also produced a cytotoxin thus masking the CT effect on Y1 cells. The production of CT in O1 and O37 as detected by VET-RPLA gave identical titres, detectable LT levels lagged behind CT. LT, CT and cytotoxin curves (titres measured in Y1 cell assay and shown in Figure 9) were different from each other. CT and the cytotoxin were first detected at 4 h, but while the CT titre levelled out at 1/320 from 8 h, the cytotoxin titre continued to rise and was 1/1280 at 24 h when the final sample was taken. The LT titre rose steadily from 1/80 at 6 h to 1/1280 at 24 h, as detected by Y1 cell assay.

To elucidate the production of CT and cytotoxin in relation to bacterial growth, the experiment was repeated using *V. cholerae* O37. The bacterial cell density was measured (OD_{621}) prior to the preparation of culture filtrates for samples taken at 2, 4, 6, 8, 10, 12, 14, 18, 20, 24 h, cytotoxin production was measured in the Y1 cell assay and CT with the VET-RPLA (Table 21 and Figure 10).

The expression of toxin (CT and cytotoxin) was found to correlate with the exponential growth phase of the organism. By late log phase of growth, 12 and 14 h post-inoculation, the production of CT peaked giving titre of 1/64 (as detected by VET-RPLA), with

Table 19 : CT production under different growth conditions.

Culture medium	Aeration/ temp.	ELISA* and VET-RPLA§					Y1 cell assay#				
		E55879 O?	1322/69 O37	WBDV-101E O49	E51116 O1	B7A *E. coli*	E55879 O?	1322-69 O37	WBDV-101E O49	E51116 O1	B7A *E. coli*
SSB	+/37	+(32)	+(64)	+(64)	+(32)	+(128)	c	c	c	2560	156250
	-/37	+(8)	+(4)	+(8)	+(4)	+(32)	c	c	c	40	6250
	+/30	+(16)	+(32)	+(32)	+(32)	+(16)	c	c	c	1280	250
	-/30	+(4)	+(4)	+(4)	+(4)	+(16)	c	c	c	40	250
SGB	+/37	+(ND)	-(ND)	+(ND)	+(ND)	+(ND)	c	-	c	40	6250
	-/37	-(ND)	-(ND)	-(ND)	+(ND)	+(ND)	-	c	c	40	31250
	+/30	+(ND)	+(ND)	+(ND)	+(ND)	+(ND)	c	c	c	1280	250
	-/30	+(ND)	+(ND)	+(ND)	+(ND)	+(ND)	c	c	c	320	1250
TSB	+/37	+(32)	-(0)	+(8)	+(32)	+(128)	c	c	c	160	156250
	-/37	-(0)	-(0)	-(0)	+(32)	-(0)	-	c	-	320	250
	+/30	+(16)	+(4)	+(4)	+(16)	+(16)	c	c	c	c	1250
	-/30	+(8)	-(0)	-(0)	+(8)	-(0)	c	c	-	40	250
BHI	+/37	+(ND)	-(ND)	+(ND)	-(ND)	+(ND)	c	c	c	20	6250
	-/37	+(ND)	-(ND)	-(ND)	-(ND)	+(ND)	c	c	-	-	1250
	+/30	+(ND)	-(ND)	+(ND)	+(ND)	-(ND)	c	c	c	40	250
	-/30	+(ND)	-(ND)	+(ND)	+(ND)	+(ND)	c	c	c	40	250
CYE	+/37	+(8)	+(8)	+(16)	+(16)	+(32)	c	c	c	160	31250
	-/37	+(4)	+(2)	+(4)	+(8)	+(32)	c	c	c	40	6250
	+/30	+(8)	+(8)	+(8)	+(8)	+(4)	c	c	c	80	250
	-/30	+(2)	+(2)	+(4)	+(2)	+(4)	c	c	c	20	250

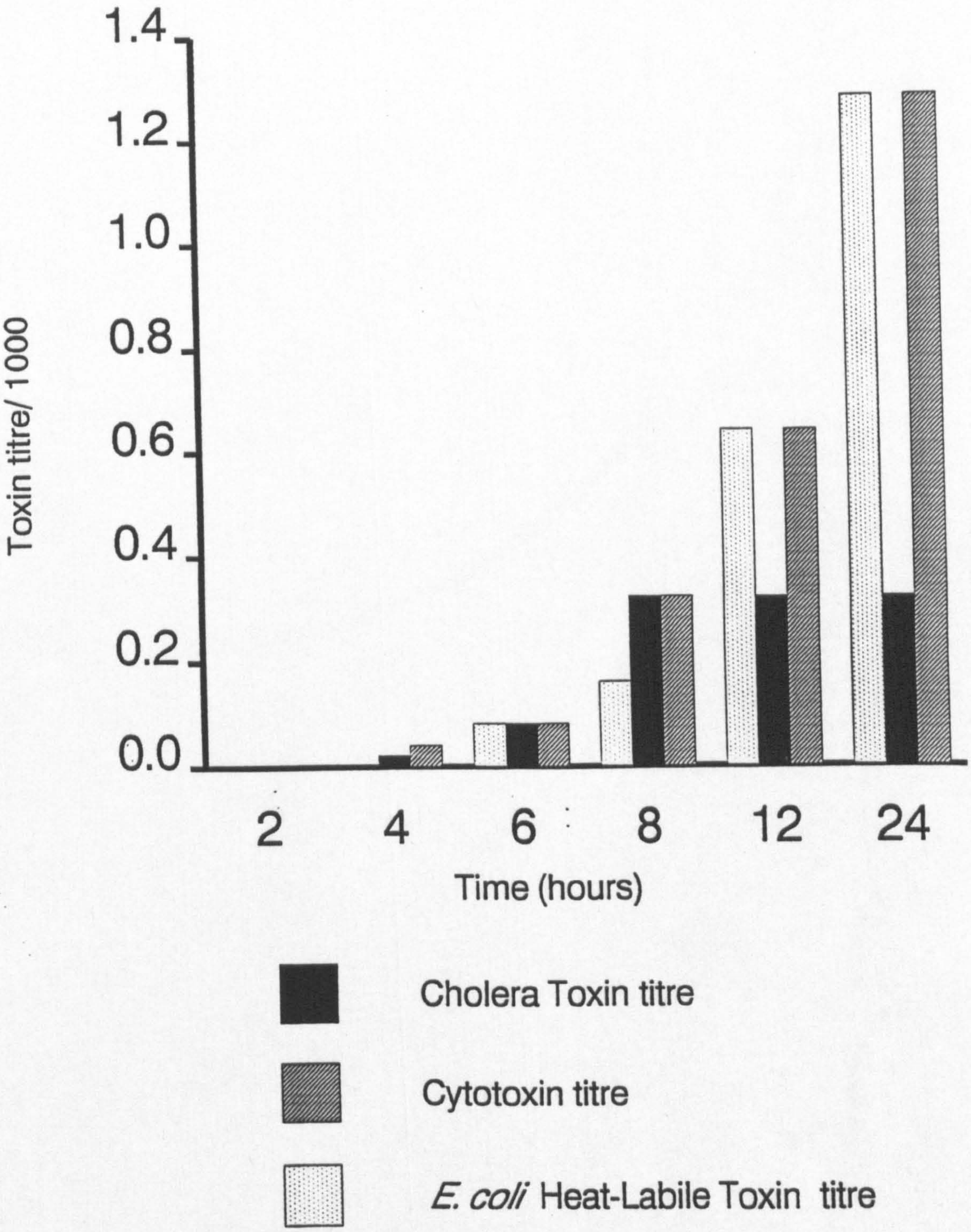
ND = not done; c = cytotoxin; *ELISA positive OD405 nm >1; § figure in parenthesis is titre of CT and LT in RPLA; # the final cytotonic titre is shown

Table 20: Kinetics of production of CT and LT

	Assay and time of sampling in hours																	
	VET-RPLA						Y1 cell assay						ELISA					
	2	4	6	8	12	24	2	4	6	8	12	24	2	4	6	8	12	24
Strain																		
E. coli B7A	-	-	-	4	4	16	-	-	80	160	640	1280	-	-	-	+/-	+	+
V. cholerae O1	-	2	4	8	16	32	-	20	80	320	320	320	-	-	+	+	+	+
V. cholerae O37	-	2	4	16	32	32	-	40c	80c	320c	640c	1280c	-	+	+	+	+	+

The titres shown are for LT (*E. coli*) and CT (*V. cholerae*) in all cases except in the Y1 assay for *V. cholerae* O37; the titres marked with a c, are cytotoxin titres.

Figure 9: Kinetics of production of CT, LT and cytotoxin

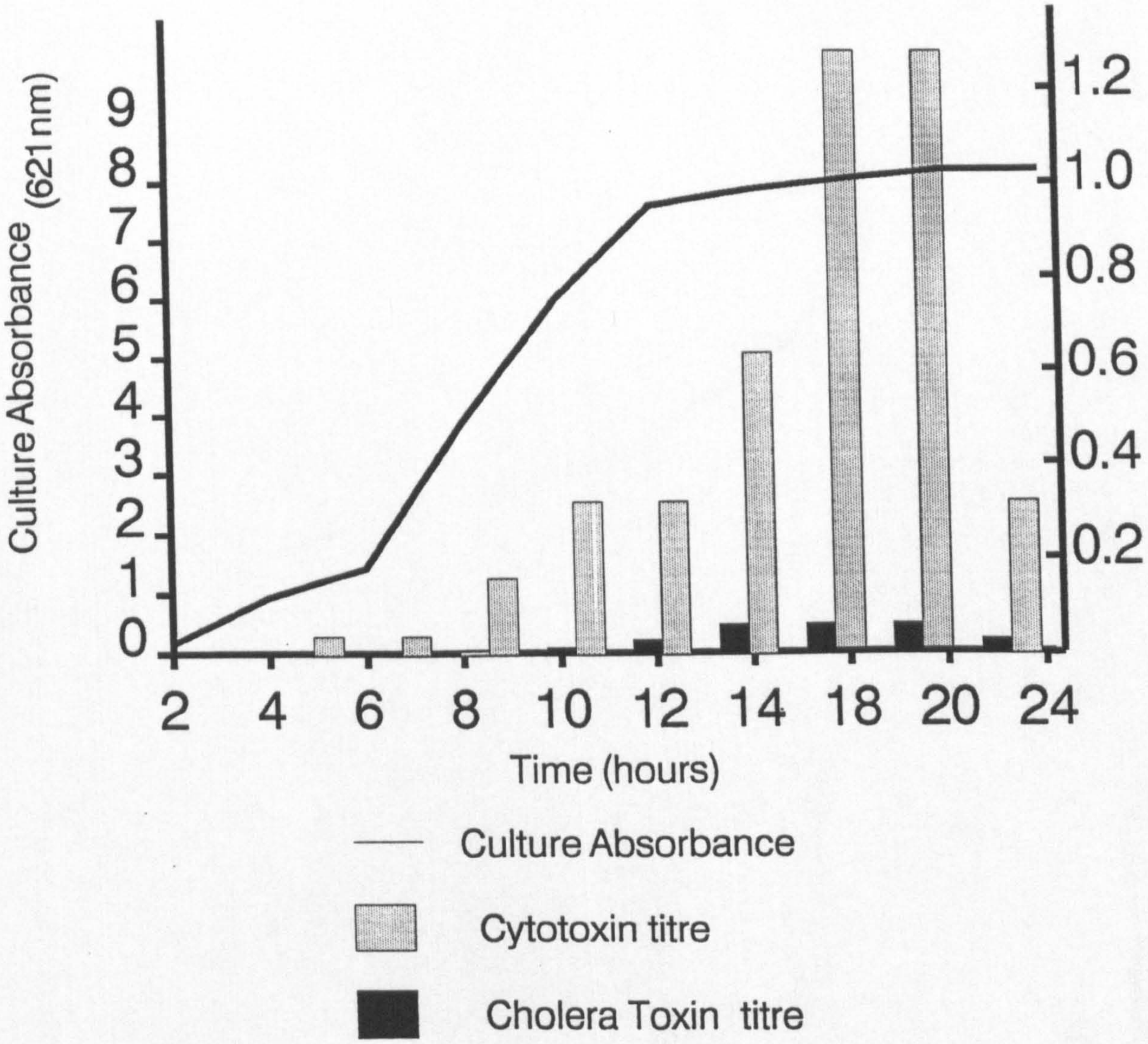


The titre of CT, LT and cytotoxin as detected by the Y1 cell assay is shown. The CT and cytotoxin titres are identical at for the first 8 h, then the cytotoxin levels continue to rise as the CT titre levels off.

Table 21: Production of CT and cytotoxin by *V. cholerae* O37; relation to the growth curve.

Time (h)	2	4	6	8	10	12	14	18	20	24
OD_{621}	0.17	0.95	1.42	3.81	5.99	7.58	7.81	7.97	8.13	8.13
VET-RLPA	0	2	4	8	16	32	64	64	64	32
Y1 cells	0	40	40	160	320	320	640	1280	1280	320

Figure 10: Production of CT and cytotoxin by *V. cholerae* O37; relation to growth curve.



The expression of CT and cytotoxin correlates with the exponential growth phase of the organisms.

a slight decrease in toxin titre by 24 h. The production of cytotoxin (detected in the Y1 cell assay) peaked at around 16 h, with a substantial drop in titre between 20 and 24 h.

3.3.3b Are the cytotoxin and CT of *V. cholerae* O37 extracellular or intracellular?

V. cholerae O37 was grown in 10 mls SSB medium as described above. After 8 h post-inoculation a 3 mls sample was aseptically removed and a sterile culture filtrate prepared, this was designated filtrate I ("extracellular toxin"). Polymixin B (1 mg/ml) was added to the remaining culture and incubated for 10 min at 37 °C, 3 mls of this was used to prepare filtrate II ("intracellular toxin"). The filtrates were titrated in the VET-RPLA (for CT) and on Y1 cells (for cytotoxin). Filtrate I gave a titre of 1/32 in the VET-RPLA test and a cytotoxic titre of 1/320 on the Y1 cells; in contrast filtrate II gave a titre of 1/2 in the VET-RPLA and was negative on the Y1 cells. The results indicated that both CT and cytotoxin were exported out of the periplasm, although there was detectable intracellular CT, the cytotoxin was not found in filtrate II.

3.3.4 Southern blot analysis of CT genes

The CT genes of probe positive *V. cholerae* non-O1 were analysed by Southern blot, to look for differences in the CT genes, by size of hybridising fragment. DNA preparations were made initially by the GES (2.3.4a) and then by the CTAB methods (2.3.4b).

The GES method was lengthy and difficult to use with *V. cholerae* and was soon replaced by the simpler and more effective CTAB method, which produced DNA preparations of good quality. The GES method was not found to have any advantages over the CTAB method, therefore the DNA preparations used for Southern blot analysis were made by the CTAB method.

DNA preparations were made of CT-probe-positive strains; *V. cholerae* reference strains O1 Ogawa biotype El Tor (10954/1), O-rough (CA385), O37 (1322-69), O105

(571-88), O115 (523-80, a *V. mimicus*), O139 (MO45), control strains *V. cholerae* O1 Ogawa biotype classical (E51116), *V. cholerae* O49 (WBDV-101E), the 5 Australian *V. cholerae* strains (2 belonging to serogroup O6 and 3 to serogroup O23, P. Desmarchelier) and 6 wild type strains of *V. cholerae* (4 belonging to serogroup O139 and 2 unidentifiable (O?)). A DNA preparation of *E. coli* B7A was also made. The DNA was digested with *Hin*dIII, run on a 1% agarose gels (2.3.4d) and Southern blots prepared (2.3.4e). The blots were probed with either CTA or CTB polynucleotide or GM1 oligonucleotide probes; the results were the same for all three probes.

The CT-positive *V. cholerae* strains could be differentiated into 7 different patterns by size of hybridising fragment (Table 22 and Figure 11). An eighth pattern (designated H5) belonged to the only CT-positive *V. mimicus* (reference strain 523-80, serogroup O115) found in this study. A single hybridising band of approximately 22 Kb, designated pattern H3, was found in 6 CT-positive *V. cholerae* non-O1, belonging to serogroups O6, O49 and O105. This band appeared to be similar or identical to the single band of *V. cholerae* O1 (10954/1). Patterns H1 - H5 had only 1 hybridising fragment of different sizes; patterns H6 and H7 had 2 hybridising fragments and pattern H8 had 3 fragments indicating the presence of more than one CT gene in these strains. Pattern H6 was exclusive to serogroup O139 (although 1 strain of serogroup O139 was designated H2 because it only possessed one of the hybridising fragments) and pattern H7 was exclusive to serogroup O23 (Table 22).

Southern blots of the *E. coli* B7A strain would only hybridise with the CTA and CTB probes at low stringency (37 °C) or the GM1 oligonucleotide at 48 °C, under these conditions a faint 2 kb hybridising fragment was found (figure 11).

Southern blot analysis and hybridisation with the GM1 oligonucleotide was also used for probe-negative, but GM1-ELISA-positive strains (3.3.2a). The GM1 oligonucleotide is directed against the part of the CT gene which determines the ability to bind GM1. The probe-negative, GM1-ELISA-positive strains produce a presumptive

Table 22: CT positive *V. cholerae* strains differentiated by Southern blot analysis

Strain	Source*	Country	Serogroup	Southern blot
1322-69	RS	Asia	O37	H1 (>25 kb)
E89007	D	India	O139	H2 (23 kb)
10954/1	RS	Asia	O1 Ogawa	H3 (22 kb)
N2	W	Australia	O6	H3
N87	W	Australia	O6	H3
WBDV-101E	D	Thailand	O49	H3
571-88	RS	?	O105	H3
E55879	D	Egypt	O?	H3
E66371	D	Egypt	O?	H3
CA385	RS	Asia	O-rough	H4 (20 kb)
523-80	RS	?	O115	H5 (17 kb)
MO45	RS	India	O139	H6 (25 and 23 kb)
E85943	D	?	O139	H6
E86270	D	Bangladesh	O139	H6
E87509	D	India	O139	H6
N7	W	Australia	O23	H7 (9 and 4 kb)
N9	W	Australia	O23	H7
N92	W	Australia	O23	H7
E51116	?	Bangladesh	O1 Ogawa	H8 (22, 20 and 17 kb)

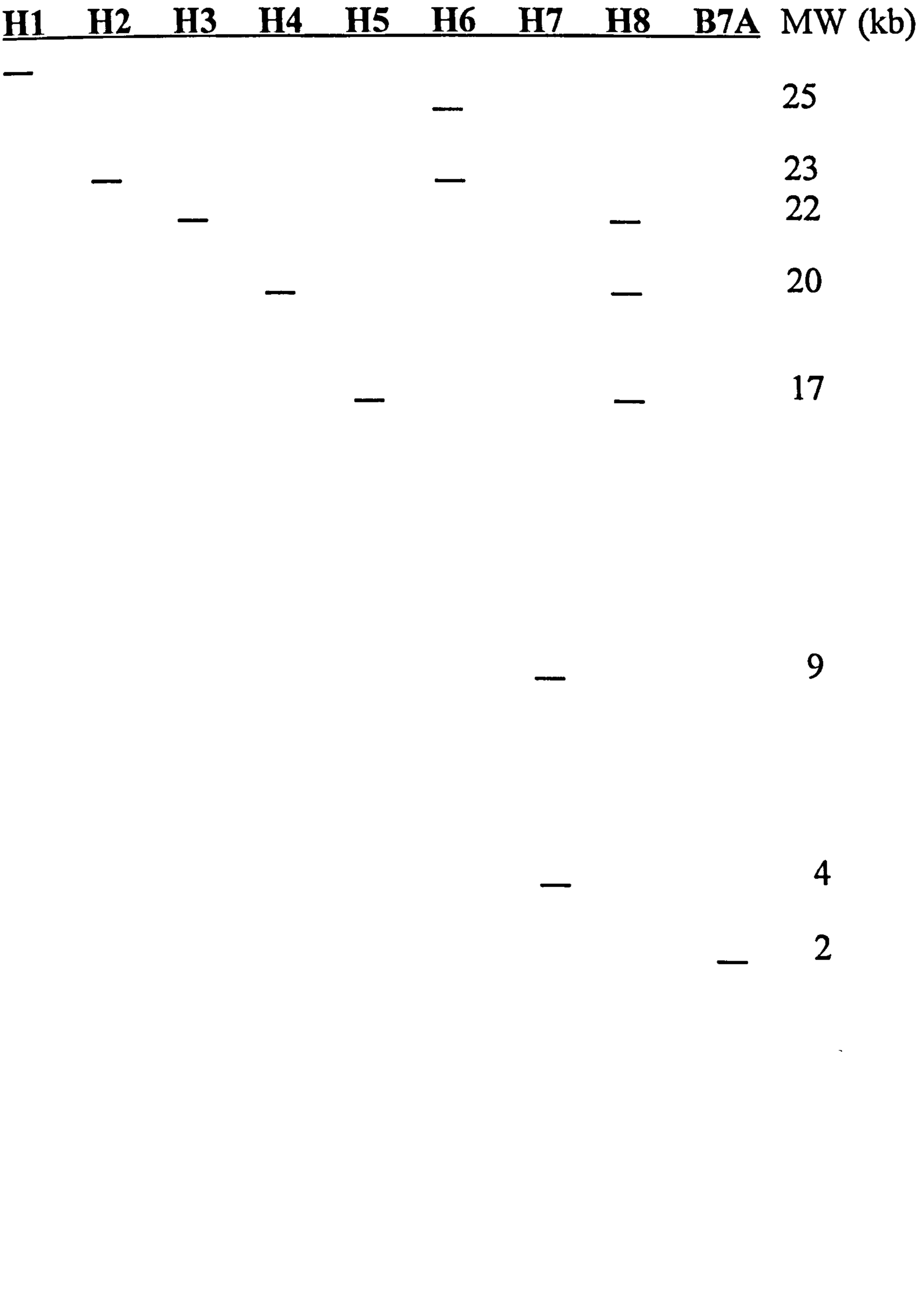
* RS = reference strain

D = strain isolated from a case of diarrhoea

W = strain isolated from water

? = source or country unknown

Figure 11: *Hin*dIII restriction digest, fragments hybridising with GM1 oligonucleotide: a diagrammatic representation.



heat-labile toxin distinct from CT but with the ability to bind ganglioside GM1, and may therefore have a DNA sequence similar to that encoded by the GM1 oligonucleotide (3.3.1b). Ten of the probe-negative, GM1-ELISA-positive *V. cholerae* non-O1 strains were tested but no hybridising fragments were detected, this was expected as colony hybridisation with the GM1 oligonucleotide was negative.

3.3.4a CT and other toxins of *V. cholerae* O139

Strains of *V. cholerae* belonging to serogroup O139 are a group of CT-producing bacteria which first emerged in India and Bangladesh in 1992/3. To date, 13 strains of *V. cholerae* O139 have been referred to the LEP, and these strains were all CT-probe-positive, ST-probe-negative and positive in the VET-RPLA and GM1-ELISA. These strains also produced a true rounding effect on Y1 cells (CT titres of 1/160 were found in the 1 day test), which could be neutralised by antibodies raised to CT. However, the neutralisation was incomplete (ie; a titre of 1/160 could be reduced to 1/20) and if the cell test was continued for 4 days (when the cytotonic effect of the CT is reversed) the presence of a cytotoxin was also detected (cytotoxic titres were also in the order of 1/160). It was thought that the cytotoxin produced by strains of *V. cholerae* O139 differed from the cytotoxin of other *V. cholerae* non-O1 examined because it does not appear to mask the CT effect.

The CT genes of *V. cholerae* O139 strains were also examined by Southern blot and hybridisation with CT and GM1 probes, as described above (3.3.4). The DNA preparations were digested with *Hin*dIII and also with *Bgl*II, *Xba*I and a combination of *Bgl*II and *Xba*I (Table 23). For comparison two *V. cholerae* O1, E51116 (classical biotype) and E51165 (El Tor biotype) both originating from Bangladesh, were also investigated.

The patterns obtained with *Hin*dIII were designated as above (3.3.4, Table 22). The patterns observed with *Bgl*II were designated; B1 (one band 7 kb), B2 (2 bands of >25 kb and 7 kb), B3 (14 kb and 7.8 kb) and B4 (7.8 kb and 7 kb). The patterns observed with *Xba*I

were designated; X1 (>25 kb), X2 (10.5 kb), X3 (7.7 and 9.3 kb) and X4 (>25 kb, 7.7 kb and 9.3 kb). When *Xba*I and *Bgl*II were used in combination as recommended by Das *et al* (Das *et al.* 1993), the result was a complex series of bands ranging from 7 kb to > 25 kb. The fainter, higher molecular weight bands, may be partial digest products (Figure 12); the two main hybridising fragments, however (7 kb and 7.8 kb) appeared to be identical or similar to the bands obtained (pattern B4) when *Bgl*II was used on its own.

The *Hin*dIII digest differentiated the *V. cholerae* O139 strains from the *V. cholerae* O1 strains. The *V. cholerae* O139 strains all belonged to the H6 pattern (2 hybridising bands of 25 and 23 kb) with the exception of E89007 which only appeared to have the 23 kb band. The *V. cholerae* O1 strains hybridised with bands of 22 kb (E51165) and 22, 20 and 17 kb (E51116). The results indicate that the O139 strains and the classical O1 strain have at least 2 copies of the CT gene.

Using *Bgl*II 8 of the 10 *V. cholerae* O139 strains gave 2 main hybridising bands of 7 and 7.8 kb (pattern B4), the exceptions were E87509, which only had 1 hybridising band at 7 kb (B1) and E87954, which had 2 hybridising bands at 7 kb and >25 kb (B2) (Figure 12). In comparison the *V. cholerae* O1 strains, E51116 and E51165, had 2 (7.8 kb and 14 kb) and 1 (7 kb) hybridising fragments, respectively. The majority of *V. cholerae* O139 strains, therefore, possessed a hybridising band which was identical or similar to the single band (7 kb) found in the El Tor strain and a hybridising band identical or similar to one of the hybridising bands (7.8 kb) found in the classical strain.

Using *Xba*I 8 of the 10 *V. cholerae* O139 strains gave a pattern (X3) which appeared to be identical to that of *V. cholerae* O1 (classical biotype), the exceptions were MO45 which appeared to have an extra hybridising fragment at >25 kb, and E87954 which only appeared to have one hybridising fragment at >25 kb.

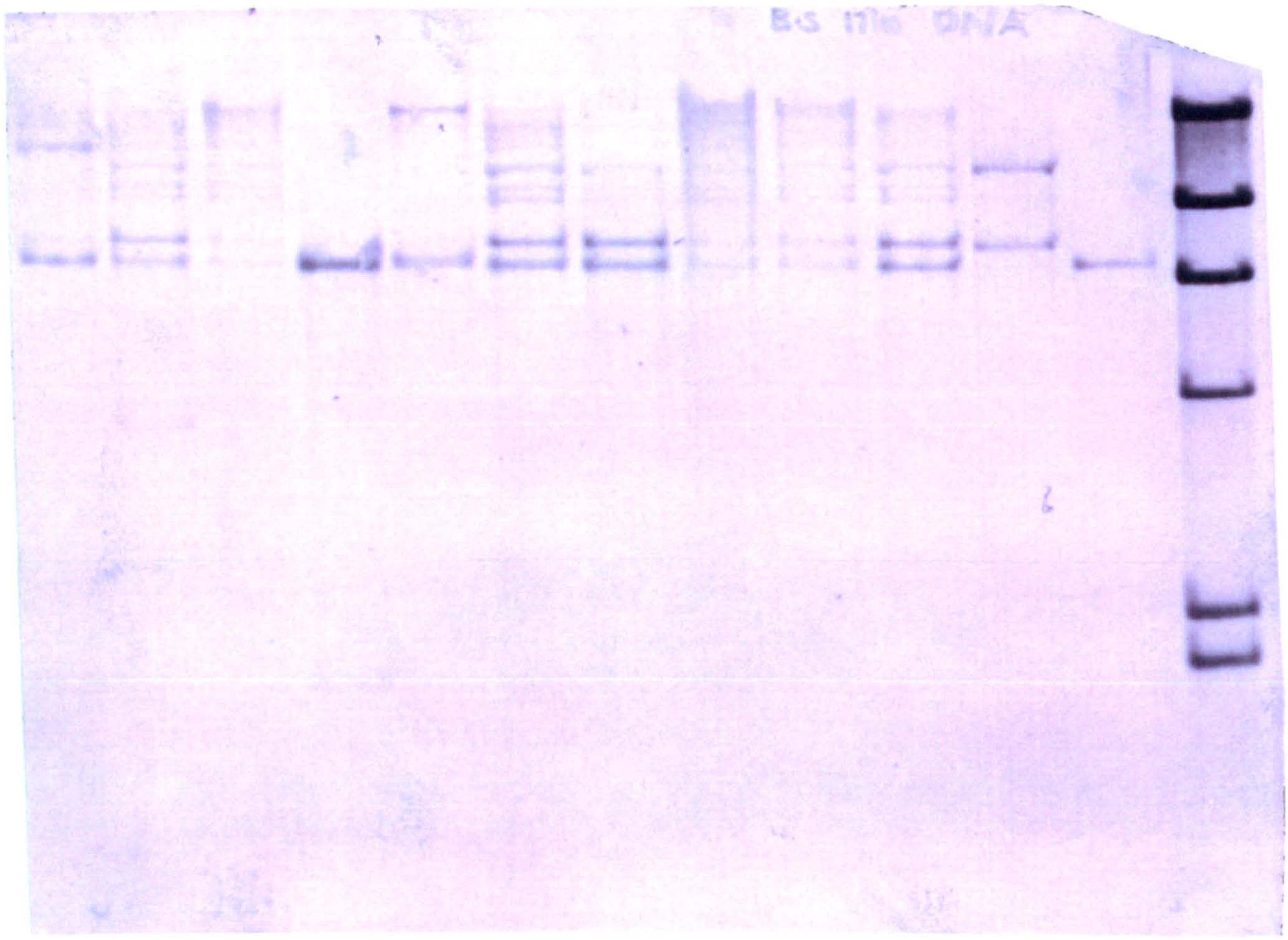
The variation in the size of hybridising fragment indicates that serogroup O139 strains are not strictly clonal in origin, however, there is no evidence to suggest that specific clones are associated with certain geographical areas.

Table 23: CT-producing strains of *V. cholerae* O1 and O139; differentiation by Southern blot analysis using *Hin*dIII, *Bgl*II, and *Xba*I.

Strain	Country of origin	*Hin*dIII	*Bgl*II	*Xba*I
V. cholerae O1				
E51116	Bangladesh	H8 (22, 20 & 17 kb)	B3 (14 & 7.8 kb)	X3 (9.3 & 7.7 kb)
E51165	Bangladesh	H3 (22 kb)	B1 (7 kb)	X2 (10.5 kb)
V. cholerae O139				
MO45	India	H6 (25 & 23 kb)	B4 (7.8 & 7 kb)	X4 (>25, 9.3 & 7.7 kb)
E85943	?	H6	B4	X3
E86270	Bangladesh	H6	B4	X3
E87509	India	H6	B1	X3
E87954	India	H6	B2 (>25 & 7 kb)	X1 (>25 kb)
E88131	India	H6	B4	X3
E89007	India	H2 (23 kb)	B4	X3
E90067	Pakistan	H6	B4	X3
E90070	Pakistan	H6	B4	X3
E90251	Thailand	H6	B4	X3

Figure 12: Southern blot analysis of CT genes

combined *Bgl*II and *Xba*I digests of CT positive *V. cholerae*



Lane 1 - 10 *V. cholerae* O139; MO45, E85943, E86270, E87509, E87954, E88131, E89007, E90067, E90070, E90251.

Lane 11 - 12 *V. cholerae* O1; E51116, classical biotype (2 bands)

E51165, El Tor biotype (1 band)

Lane 13 MW markers (kb) 23, 9.4, 6.5, 4.3, 2.3, 2.0.

3.3.5 PCR for CT

See section 3.7.1.

3.4 Detection of strains producing heat-stable toxin (NAG-ST)

3.4.1 Infant Mouse assay

This assay was used successfully to detect STA produced by strains of *E. coli* and has also been reported for NAG-ST producing *V. cholerae* non-O1 (Arita *et al.* 1986). The filtrates prepared in section 3.2.1 for reference strains of *V. cholerae* O2 - O40 were tested in the infant mouse assay (2.3.1c); the intestine weight/body weight ratios obtained were all negative (between 0.042 and 0.078), suggesting that ST toxin was not expressed by the strains of *V. cholerae* examined. These filtrates had been prepared after growth in TSB (following the protocol for *E. coli* ST). Brain Heart Infusion broth containing 0.5% NaCl was also used to growing test strains *V. cholerae* and *E. coli* prior to performing the infant mouse test. The strain of *E. coli* was positive for ST toxin in all three media giving ratios of 0.108 (TSB), 0.116 (BHI) and 0.095 (BHI+0.5% NaCl). The *V. cholerae* non-O1 strain (NAG-82E) gave indeterminate ratios (there was no obvious fluid accumulation seen) of 0.076 (TSB), 0.078 (BHI) and 0.080 (BHI+0.5% NaCl). As the use of BHI+0.5% NaCl did not yield positive results with the *V. cholerae* NAG-ST control strain, in this study, it was decided to try a different approach.

3.4.2. DNA probes for detecting strains carrying genes for ST

3.4.2a SNAP-ST

The production of NAG-ST by strains of *V. cholerae* non-O1 could not be demonstrated (3.4.1), however, strains of *V. cholerae* non-O1 could be screened for the

presence of the NAG-ST gene. The first probe used was a commercially available SNAP (synthetic nucleic acid probe) developed for the detection of *E. coli* ST (2.3.2f). The reference strains of *V. cholerae* belonging to serogroups O2 - O83, the O-rough reference strain, the NAG-ST control strain of *V. cholerae* non-O1 (NAG-82E), and 89 strains of *V. cholerae* non-O1 (44 from human and 45 from environmental sources) were tested with the SNAP-ST probe. None of the strains of *V. cholerae* examined, gave a positive reaction with the SNAP-ST test (an ST-positive *E. coli* strain was positive with this probe).

3.4.2b Digoxigenin-labelled ST oligonucleotides

Since the infant mouse and the SNAP-ST tests did not detect strains of *V. cholerae* producing ST or carrying genes encoding ST, it was decided to examine additional probes to examine strains of *V. cholerae* non-O1. Oligonucleotides were synthesised based on sequences published by Hoge *et al* (Hoge *et al.* 1990) and Ogawa *et al* (Ogawa *et al.* 1990). The two oligonucleotides described by these workers were similar in being 23 and 24 bases in length respectively, but varied by 4 bases (2.3.2g). Small scale hybridisation experiments (quarter membranes, spotted with 8 *V. cholerae* non-O1, the NAG-ST control strain of *V. cholerae* non-O1 (NAG-82E) and an ST-positive *E. coli*) were performed at 50 °C, 55 °C, 60 °C and 65 °C.

Hybridisation experiments using the probe described by Hoge *et al* were not satisfactory because the *V. cholerae* NAG-82E control strain was not positive at any of the temperatures and the results with other strains of *V. cholerae* non-O1 were difficult to read. In contrast, the probe described by Ogawa *et al* gave a clear, unequivocal positive result at 60 °C and 65 °C (a dark purple colour), and weak positive results at 50 °C and 55 °C, with the *V. cholerae* NAG-82E control strain. The optimal hybridisation temperature for the Ogawa probe was 65 °C, this gave clear purple coloured positive reactions without much background colour. For all oligonucleotide hybridisations it was essential to obtain good lysis of the *V. cholerae*

colonies on the membranes, hence the 20 min lysis described in 2.3.2d, rather than the 10 min recommended for *E. coli* colonies. The maintenance of a high probe concentration (50 ng/ml for each 50 cm^2 membrane) was also important in getting clear results.

Of 637 strains of *V. cholerae* non-O1 tested 19 (3%) hybridised with the Ogawa NAG-ST oligonucleotide. None of 11 strains of *V. cholerae* non-O1, which were previously positive for CT, were NAG-ST-positive. All 88 strains of *V. cholerae* O1 (including reference strains NCTC 10954/1 and NCTC 8457/5 and control strain E51116) were also NAG-ST probe-negative. Of 246 *V. cholerae* non-O1 strains from environmental sources 13 (5%) were NAG-ST probe-positive, whereas of 391 clinical isolates only 6 (1%) were NAG-ST probe-positive. There also appeared to be a correlation with serogroup O14; all 7 *V. cholerae* strains belonging to the O14 serogroup (4 strains from the environment and 3 strains of human origin) were NAG-ST probe-positive. In contrast to the O14 serogroup, NAG-ST probe-positive strains were the exception in other serogroups. The other NAG-ST probe-positive strains were: 6 unidentifiable (O?) strains, one each of serogroups, O6, O18, O37, O51, O58 and an O-rough strain (Table 24).

A total of 60 *V. mimicus* strains was also tested for hybridisation with the NAG-ST probe, this comprised 9 reference strains and 51 wild type strains (3 from clinical sources and the 48 from environmental sources). Seven (11.7%) *V. mimicus* strains hybridised with the NAG-ST probe, these are listed in Table 24. Of the NAG-ST probe-positive strains, 3 were reference strains (serogroups O20, O101 and O116) and 4 were isolated from prawns, with 2 of the isolates originating from India (for the other 2 isolates the country of origin was not known). The 3 strains from clinical sources, all were NAG-ST probe-negative (2 strains (E63453 and E82245) were unidentifiable (O?), and 1 (E54122) belonged to serogroup O20). There were also 3 *V. mimicus* strains which belonged to serogroup O14, however, in contrast to the *V. cholerae* O14 strains, these were NAG-ST probe-negative.

3.4.3 Stability of the NAG-ST gene.

As there were NAG-ST probe-positive and probe-negative representatives from the serogroups O6, O18, O37, O51 and O58, the stability of the NAG-ST characteristic was investigated. To determine stability of the NAG-ST gene in *V. cholerae* non-O1, positive representatives of the strains belonging to serogroups O14 (E54931), O37 (E70397) and O58 (E85071) and probe-negative representatives of O58 (E85055, E85056, E850720) and O37 (E70398) were tested. Colonies were replicated from spread plates (approximately 100 colonies per plate) onto nylon membranes and these were processed as described (2.3.2d), and hybridised with the Ogawa NAG-ST probe. No individual colonies were found which varied from parent strain with respect to the NAG-ST characteristic.

3.4.4 Southern blot analysis of the NAG-ST gene.

Southern hybridisation analysis was used to differentiate the NAG-ST probe-positive *V. cholerae* non-O1 strains, by hybridising fragment size. Genomic DNA preparations were made by the CTAB method (2.3.4b) and digested with the RE *Hin*dIII. The Southern blots were hybridised with the NAG-ST oligonucleotide probe of Ogawa *et al* and found to hybridise with a single chromosomal restriction fragment after digestion with *Hin*dIII. The size of the restriction fragment varied from 2.2 kb to 19 kb (Table 25 and Figure 13). Strains gave single bands of different sizes, with the exception of an unidentifiable (O?) *V. cholerae* strain (E47276) that had 2 hybridising fragments (8 kb and 2.3 kb), this strains appears, therefore, to have 2 copies of the NAG-ST gene. Further evidence for E47276 possessing 2 gene copies was obtained by repeating the digest and Southern blot using a different RE, *Pst*I; this confirmed that there were 2 hybridising fragments present. The size of the hybridising fragment allowed the NAG-ST probe-positive strains of *V. cholerae* to be differentiated into 8 groups (Table 25). The fragment with the highest molecular weight (19 kb), designated H1, was found in all

Table 24: List of NAG-ST gene positive strains of *V. cholerae* non-O1 and *V. mimicus*

(Including reference strains and control strains).

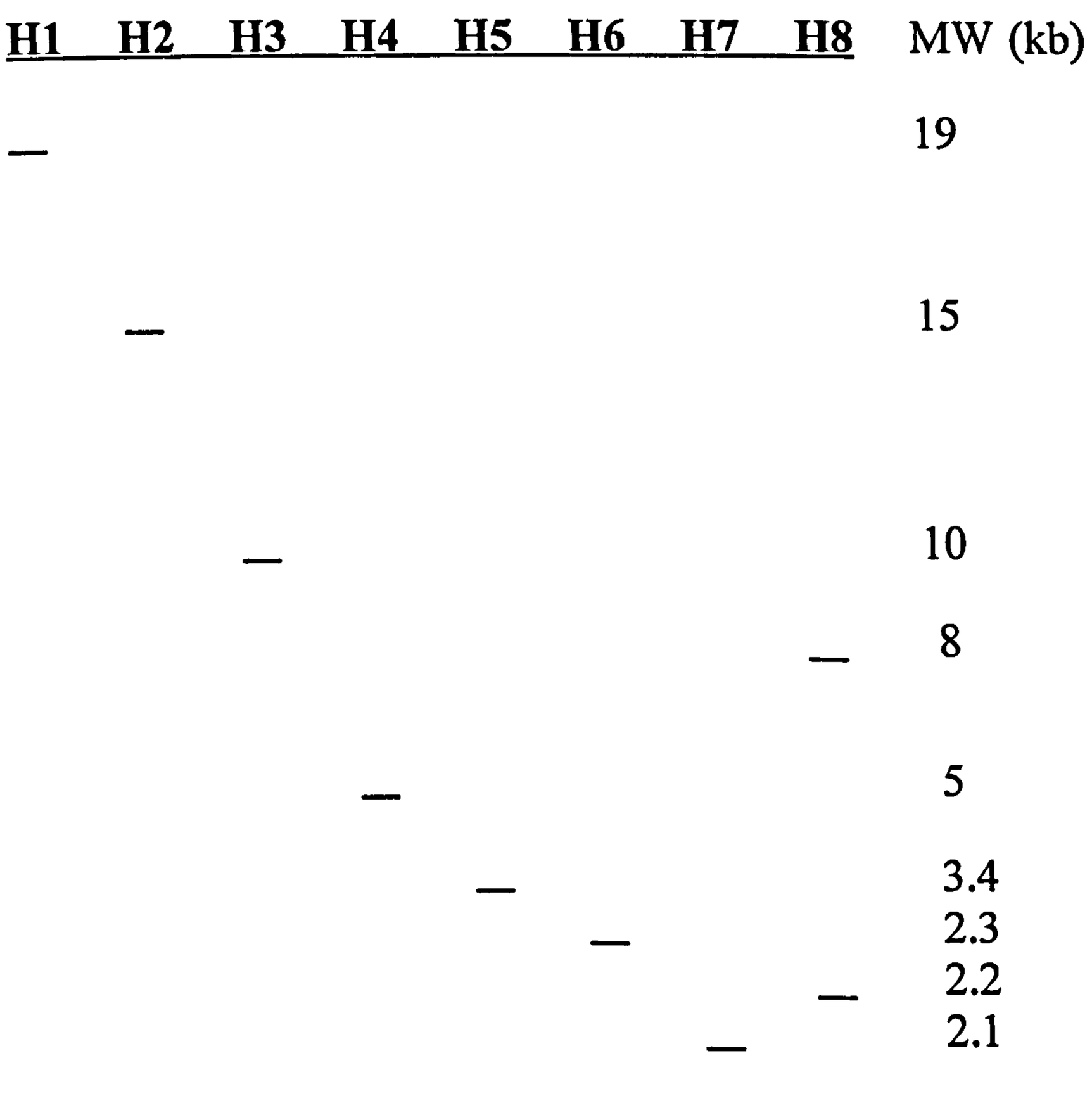
Reference No.	Serogroup	Country of origin	Patient details diarrhoea/age/sex* or source of strain
V. cholerae strains			
E54933	O-rough	Italy	water
E54873	O6	Italy	water
NAG-82E	O14	?	control strain
E49108	O14	Malta	D/52/F
E51711	O14	?	prawn
E51712	O14	?	prawn
E54931	O14	Italy	water
E54947	O14	Italy	water
E70003	O14	Pakistan	D/35/M
E89755	O14	India	D/23/M
E52779	O18	?	prawn
E70397	O37	?	prawn
E82290	O51	Tunisia	D/46/F
E85071	O58	?	water
563-88	O102	?	reference strain
E47169	O?	?	prawn
E47276	O?	?	prawn
E51707	O?	?	prawn
E72253	O?	Chile	water
E89693	O?	Tunisia	D/54/F
E90680	O?	Tunisia	D/41/M
V. mimicus strains			
10332-62	O20	?	reference strain
559-88	O101	?	reference strain
980-78	O116	?	reference strain
E57204	O?	?	prawn
E57205	O?	?	prawn
E57591	O?	India	prawn
E76337	O?	India	prawn

* Patient details; ? = unknown, D = diarrhoea, age = stated in years, M = male, F =female

Table 25: Heat-stable enterotoxin (NAG-ST) oligonucleotide positive *V. cholerae* non-O1 strains differentiated by Southern blot analysis

Strain	Serogroup	Southern blot
NAG8-2E	O14	H1 (19 kb)
E49108	O14	H1
E70003	O14	H1
E89755	O14	H1
E51711	O14	H1
E51712	O14	H1
E54931	O14	H1
E54933	O-rough	H1
E54947	O14	H1
E47169	O?	H2 (15 kb)
E51707	O?	H2
E72253	O?	H3 (10 kb)
E52779	O18	H4 (5 kb)
E54873	O6	H5 (3.4 kb)
E90680	O?	H5
E70397	O37	H6 (2.3 kb)
E82290	O51	H6
E89693	O?	H6
E85071	O58	H7 (2.1 kb)
E47276	O?	H8 (8 kb and 2.2 kb)

Figure 13: *Hin*dIII restriction digest of *V. cholerae* non-O1, fragments hybridising with NAG-ST (Ogawa) oligonucleotide: a diagrammatic representation.



strains of serogroup O14, including the NAG-82E control strain, and an O-rough strain. This O-rough strain may have derived from an O14 strain as the H1 pattern was otherwise exclusive to serogroup O14. The H1 pattern was found regardless of source or geographical origin, indicating that the *V. cholerae* O14 serogroup is a single clone. The other *V. cholerae* non-O1 strains belonged to Southern blot patterns as shown in Table 25, these patterns did not appear to correlate with serogroup.

3.5 Detection of strains producing Vero-cytotoxin

3.5.1 Tissue culture (Vero cell) assays for VT.

Toxins expressed by strains of *V. cholerae* non-O1 affect Vero cell monolayers in two ways (see also section 3.2.1a). Firstly, the toxin(s) can have a cytotoxic effect, resulting in the detachment of the entire monolayer of Vero cells, or they can cause a vacuolation effect (3.2.1a, figure 6). Vero cells have principally been used to detect VT produced by strains of *E. coli* (VTEC) and the production of a VT-like toxin by *V. cholerae* non-O1 has been reported (O'Brien *et al.* 1984). However, the cytotoxic effect observed on Vero cells in this study was not neutralised by antibodies to VT.

3.5.2 DNA probes for detecting genes encoding VT.

Although VT production could not be detected on Vero cells, 150 strains of *V. cholerae* non-O1 and 10 strains of *V. mimicus* were examined for the presence of VT genes using a combined VT1 and VT2 polynucleotide probe (2.3.2b). None of the strains tested hybridised with the VT1+2 probe.

3.6 Ability of strains to produce Haemolysin

Strains of *V. cholerae* and *V. mimicus* were examined for the ability to lyse red blood cells using agar plates containing 1% horse blood, a strain was considered as haemolytic when a readily observable zone of haemolysis was present around bacterial colonies. Haemolysis of red blood cells other than those from horse, calf, sheep, human and rat, were tested using a microtitre method. All the strains used in this study (Appendix 2) were tested on horse blood agar. Of the strains of *V. cholerae* non-O1 and *V. mimicus* tested, 90% of both species were haemolytic on horse blood agar. Haemolytic strains generally produced ß-haemolysis (complete lysis, clearing, on horse blood agar), with zone sizes varying from > 5 mm to < 1 mm. Many strains also produced α-haemolysis (greening on horse blood agar) and a possible third haemolysin was observed with 5% of strains, with haemolysis appearing as a double zone.

Haemolysins are heat-labile toxins which are secreted by strains of *V. cholerae* and *V. mimicus* and can be detected in culture filtrates prepared as previously described from organisms grown in Syncase sucrose broth (2.3.1a). Fifty strains of *V. cholerae* non-O1 were examined for the quantitative expression of a haemolysin active on rat rbcs, using a microtitre method. Volumes of culture filtrate (50 µl) were diluted, by doubling dilution in saline, in a 96-well tissue culture plate. An equal volume of 1% washed rbcs was added to each well and the plate was incubated at 37 °C for 1 h and then at 4 °C overnight. The titre was measured by noting the lowest dilution at which haemolysis was present. In the microtitre system if haemolysin was produced there was no cell pellet and the well was a red/brown colour due to the release of haemoglobin from the ruptured cells; if there was no haemolysin production the intact rbcs formed a tight pellet and the colour of the liquid in the well was clear to pale orange (Figure 14). Titres ranged from 1/32 to 1/128 and five strains were negative for haemolysis on rat blood, even though all but one of these strains were all ß-haemolytic on horse blood agar plates. The culture filtrates of these strains were also tested in Y1 tissue culture cells and there

was no apparent correlation of haemolysin with cytotoxic effects in cell tests. It was found that strains which did not express a haemolysin produced a cytotoxin causing a ghosting effect on Y1 cells, whilst culture filtrates, prepared from strains producing haemolysin, did not affect the appearance of Y1 cells.

The ability of 8 ß-haemolytic strains of *V. cholerae*, belonging to serogroups associated with clinical disease (O2, O5, O34 and O37), to lyse rbcs from other animals, was further investigated. The 8 strains were; E38820 (O2), E54117 (O5), E60150 (O34), E54418 (O37) and the reference strains for these serogroups NCTC 4711 (O2), B4202-64 (O5), 152-68 (O34) and 1322-69 (O37). Rat, guinea pig, calf, sheep and a human rbc's were tested using the microtitre method. All strains tested produced the highest titre of haemolysin with rat rbcs, with decreasing titres with guinea pig, followed by human and then bovine and then sheep. The reference strain of *V. cholerae* for serogroup O34 did not produce detectable haemolysis with calf or sheep rbcs. These same strains were used to investigate whether haemolysin produced by *V. cholerae* non-O1 was iron-regulated (see 3.6.2).

3.6.1 Purification of haemolysin produced by *V. cholerae* O2.

The reference strain of *V. cholerae* for serogroup O2 (NCTC 4711) was used to determine optimum growth conditions for the production of haemolysin, with the aim of purifying and raising antiserum to this haemolysin. It was envisaged that the antiserum could be used to investigate the haemolysin(s) produced by other *V. cholerae* non-O1 and the closely related *V. mimicus*. An antiserum would also be useful in elucidating the relationship, if any, between haemolysin(s) and cytotoxin(s).

To determine optimal growth conditions for haemolysin production *V. cholerae* O2 (NCTC 4711) was grown in Syncase sucrose broth (SSB) at 30 °C and 37 °C (both static and with aeration). The culture filtrates were tested in the microtitre system with 1% rat blood, as described above (3.6). The highest titre (1/64) was obtained at 37 °C, with

aeration. *V. cholerae* O2 (NCTC 4711) was also grown in different growth media at 37 °C, with aeration, these growth media were; Trypticase soy broth (TSB), Brain heart infusion broth (BHI), Nutrient broth (NB), Tris succinate medium (TS) and SSB containing 1 mg/ml desferal. Since the expression of certain haemolysins is regulated by the availability of iron, Tris succinate medium (TS) and SSB containing 1 mg/ml desferal were also used (see also sections 3.6.2 and 3.12). Haemolysin production was reduced to levels below detection when *V. cholerae* O2 was grown in SSB + 1 mg/ml desferal. Culture filtrates from TSB and BHI both had haemolytic titres of 1/16, filtrates from SSB and TS medium both had haemolytic titres of 1/64 and filtrates from NB gave the highest haemolytic titre at 1/128. Haemolysin production appeared to be inoculum dependent; haemolytic titres were increased at least two-fold if a starter culture (an overnight PW) was used and diluted 1/10 in fresh NB and the strain grown at 37 °C with aeration for 6 - 8 h. The haemolytic titre in relation to the growth curve of *V. cholerae* O2 is shown in Figure 15. 20 mls NB was inoculated with 2 mls of starter culture, 0.5 mls samples were removed every hour and the OD_{621} taken as a measure of cell density, the sample was centrifuged and filtered through a 0.2 μm Millipore filter and tested for haemolytic activity in microtitre with 1% rat rbcs (Table 26). The haemolysin peak occurred at 5 - 6 h, followed by a decline in detectable haemolysin coinciding with the end of the log phase of growth. The loss of haemolytic activity may be due to the release of proteases and other enzymes as cell death occurs (Figure 15).

The optimal conditions described above, 2 mls of starter culture in 20 mls NB at 37 °C with aeration for 6 h, were used to produce a culture filtrate for toxin purification. Haemolysin was partially purified using by passing the crude toxin preparation through a chromatography column containing Sephadex G200. Eluted toxin was detected using 1% rat blood in a microtitre assay, and the positive fractions (25, 26, 27 and 28; wells A2-A5 in Figure 14) were pooled and lyophilised. The procedure was repeated to obtain at least three batches of lyophilised partially purified haemolysin. An antiserum was prepared to the crude

Figure 14: Haemolysis of rat red blood cells by *V. cholerae* non-O1 detected using a microtitre assay.

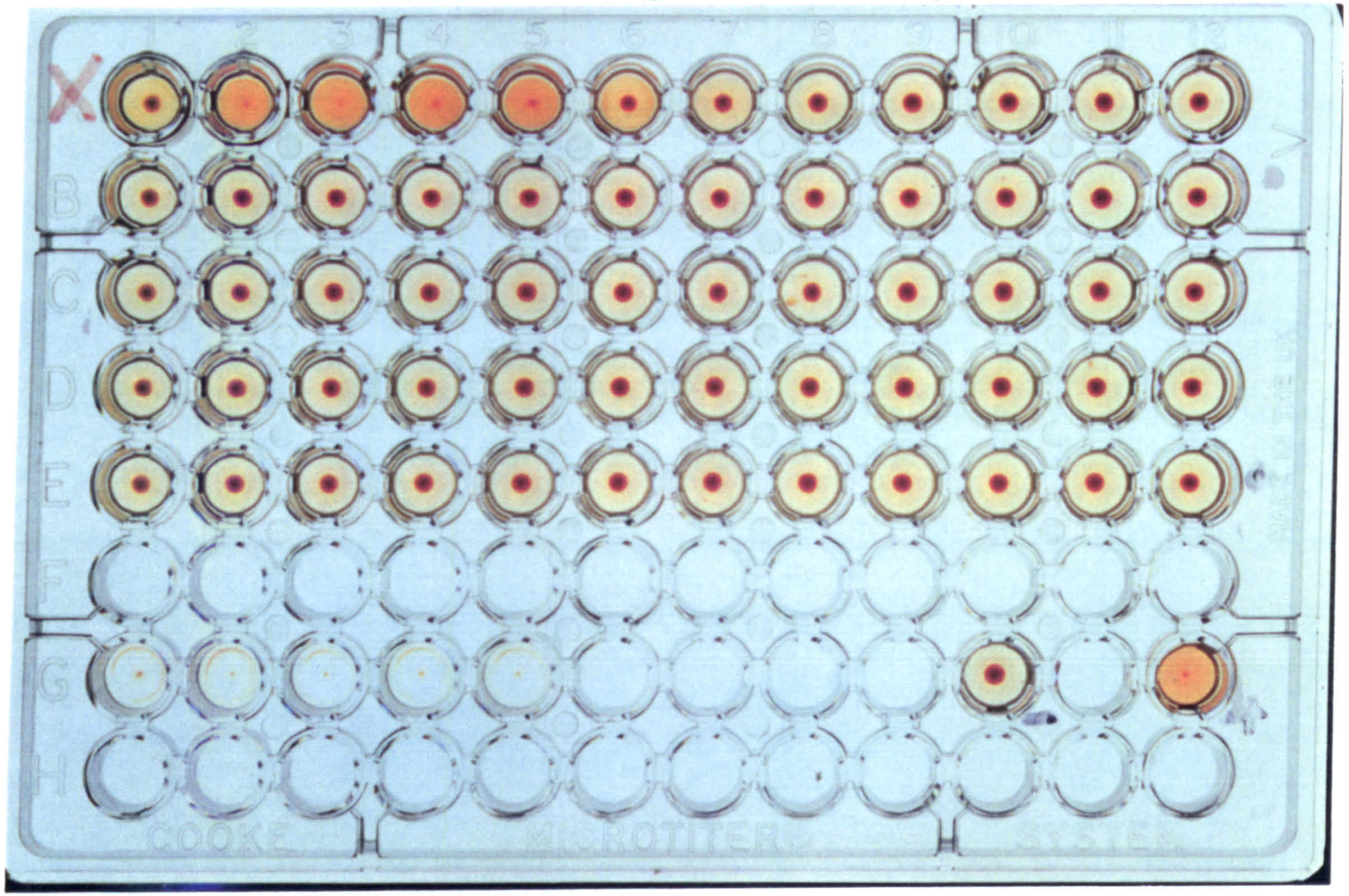
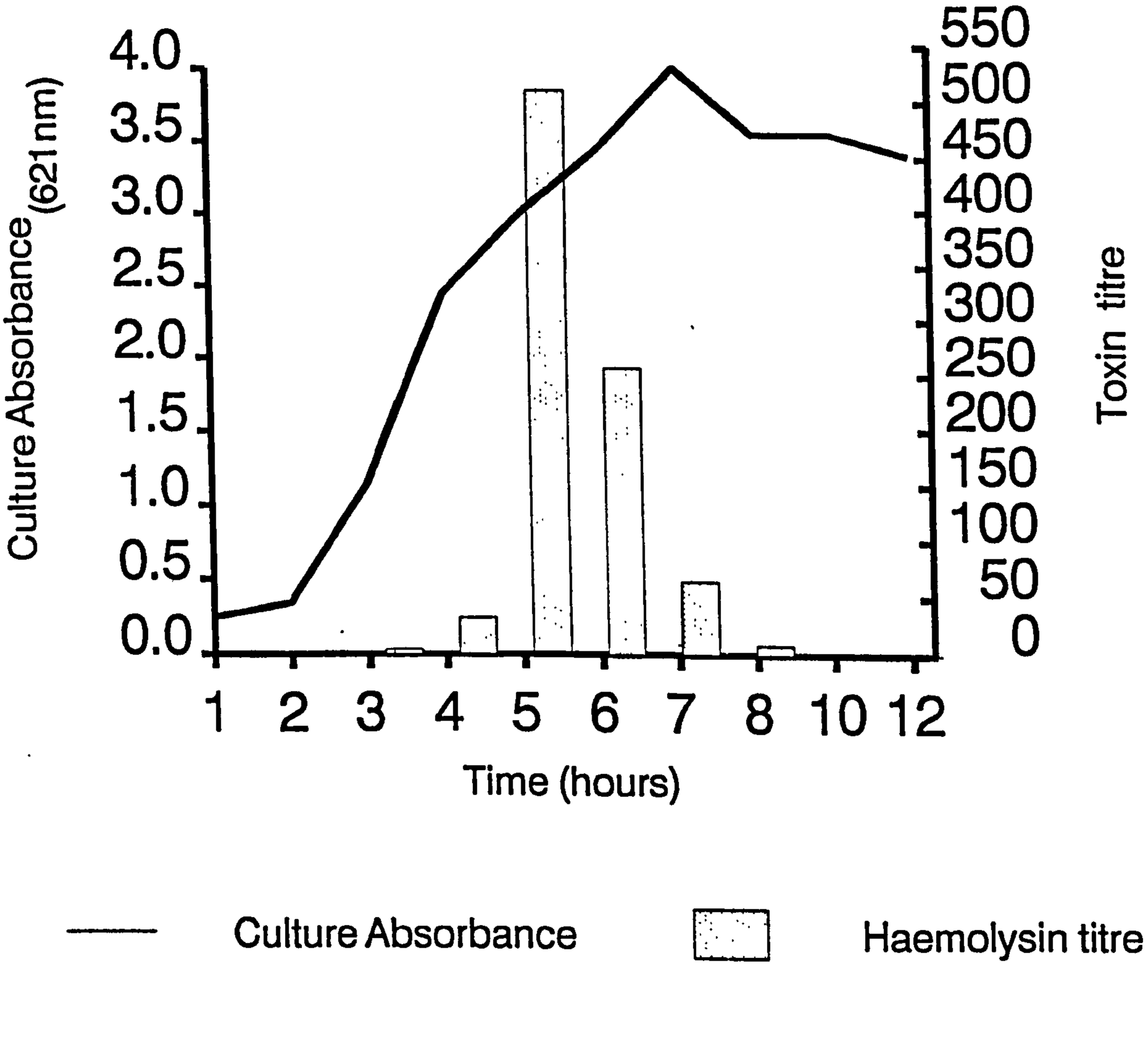


Table 26: Production of haemolysin by *V. cholerae* O2; relation to the growth curve.

	Time (h)									
	1	2	3	4	5	6	7	8	10	12
OD_{621}	0.25	0.35	1.15	2.45	3.00	3.45	4.00	3.55	3.55	3.40
Haemolytic titre	2	2	4	32	512	256	64	8	0	0

Figure 15: Production of haemolysin by *V. cholerae* O2; relation to growth curve.



The expression of haemolysin correlates with the exponential growth phase of *V. cholerae* O2. The haemolytic activity declines as cell death occurs, possibly due to the release of proteases and other enzymes.

toxin using a New Zealand White rabbit (animal work was performed under Home Office licence PPL 70/03323). One of the three batches of toxin preparation was dissolved in 0.25 mls distilled water and mixed with 0.25 mls Freund's complete adjuvant, emulsified with brief sonication. The first 0.5 mls injection was administered intra-dermally, followed 20 days later by two 0.25 mls intra-muscularly, on day 31 a test bleed was taken. A pre-bleed and the test bleed were tested for the presence of neutralising antibody (25 µl antiserum double diluted in saline in a microtitre plate was incubated with 25 µl of filtrate for 3 h at 37 °C, then 50 µl 1% rat blood was added). As expected the pre-bleed serum did not neutralise haemolysis, however the test bleed serum also had no effect on haemolysis. The rabbit was boosted with toxin preparation in 0.5 mls distilled water intra-venously on day 33 and a second test bleed taken on day 45, the serum from this test bleed also showed that no antibodies had been raised against the haemolysin. A final 0.5 mls intra-venous injection was given and a test bleed taken on day 67, unfortunately this too was negative for antibodies to haemolysin and the experiment was terminated on day 77.

3.6.2 The role of iron in the regulation of expression of *V. cholerae* haemolysin

Expression of haemolysins by certain strains of, for example, *E. coli* has been shown to be iron-regulated. In this section of the study, the role of iron in the ability of different serogroups of *V. cholerae* to produce haemolysin was examined (see also 3.12). Eight ß-haemolytic strains of *V. cholerae* belonging to serogroups associated with clinical disease (O2, O5, O34 and O37) which were examined (section 3.6) for haemolysis on different rbcs, were also used for these experiments. Test strains, E38820 (O2), E54117 (O5); E60150 (O34), E54418 (O36) and the reference strains for these serogroups were grown in SSB + 1 mg/ ml desferal and in Tris succinate medium. Strains grew poorly or not at all in the presence of desferal; in contrast, bacteria grew comparatively well in Tris succinate and this medium was used to provide iron limited conditions for subsequent experiments. Bacteria were grown in

Tris succinate medium and siderophores detected as described (2.3.8). Although strains of *V. cholerae* non-O1 grew in Tris-succinate medium, these bacteria grew particularly well in the presence of added iron (5 μg $FeCl_3$/ ml) at 37 °C with aeration. Growth in Tris-succinate medium resulted in the expression of the siderophore enterochelin; however, under these culture conditions haemolysin was not produced. When the same strains were grown statically in Tris-succinate medium with added iron (5 μg/ml $FeCl_3$), enterochelin was not produced but haemolysin was detected. An exception to this was *V. cholerae* strains E38820 and E54117 which produced both enterochelin and haemolysin (Table 27). Reference strain 1322-69 (O37), a CT probe-positive strain, did not grow, and reference strain 152-68 (O34) grew very poorly in Tris-succinate medium, even with the addition of $FeCl_3$, and it was not possible to detect haemolysin production (or enterochelin for the O37 strain) under these conditions. Both strains produced haemolysin under optimal conditions. Based on these results production of haemolysin appears not to be iron regulated.

3.7 Polymerase chain reaction (PCR)

PCR was used for the detection of the cholera toxin gene, *ctxB*, and for the detection and differentiation of the toxin co-regulated pilus gene, *tcpA*.

The PCR for *ctxB* was used with CT-probe-positive strains, to determine whether the CT gene could be differentiated by CT-PCR and with CT-probe-negative, GM1-ELISA-positive strains, to determine if gene sequences similar to CTB could be detected by these primers. The strains examined by *ctxB* PCR were 22 CT-probe -positive *V. cholerae* non-O1 (Table 28), 12 CT-probe-negative, but GM1-ELISA-positive strains (of which 3 are included in Table 28, 8457/5 (O1), E54418 (O37) and 1154-74 (O49)) and the only CT-probe-positive *V. mimicus* (reference strain O115).

The PCR for *tcpA* has been used to differentiate the *tcpA* of the 2 biotypes of *V. cholerae* O1 (El Tor and classical), in this study the PCR for *tcpA* was used to determine if

Table 27: Enterochelin and haemolysin production (titre/OD_{621}) for strains of *V. cholerae* non-O1

Ref. No.	Serogroup	shaken/unshaken	enterochelin titre OD_{621}	haemolysin titre OD_{621}
NCTC 4711	O2	S	0.015	-
		U	-	25.8
E38820	O2	S	0.016	-
		U	0.040	7.55
B4202-64	O5	S	0.027	-
		U	-	29.63
E54117	O5	S	0.033	-
		U	0.027	5.41
152-68	O34	S	0.031	-
		U	0.007	-*
E60150	O34	S	0.085	-
		U	0.076	2.17
1322-69	O37	S	ND	ND
		U	ND	ND
E54418	O37	S	0.042	-
		U	0.037	40.00

* 152-68 (O34) grew poorly and no haemolysin was detected.

ND = not done, because 1322-69 (O37) did not grow in this medium.

tcpA sequences were present in all CT-probe-positive *V. cholerae* non-O1 and if these sequences could be differentiated. The strains used for the *tcpA* PCR were; 34 *V. cholerae* non-O1, 22 of which were CT-probe positive, and 3 *V. cholerae* O1 strains of which 8 were CT-probe positive. The strains were selected because they were CT-probe-positive or because they were CT-probe-negative strains representatives belonging to the same serogroups (Table 28). There were 3 CT-probe-negative, GM1-ELISA-positive strains; 8457/5 (O1), E54418 (O37) and 1154-74 (O49). The CT-probe-positive *V. mimicus* was also examined by the PCR for *tcpA*. For all PCR assays the annealing temperature was 55 °C and the procedure was that described in section 2.3.3. The expected sizes of the amplified fragments were 460 bp for *ctxB* and 617 bp and 471 bp for *tcpA* (classical and El Tor, respectively). The three sets of primers (2.3.3) were not used simultaneously in the same reaction, because it was difficult to distinguish the 460 bp *ctxB* and the 471 bp El Tor *tcpA* amplicons which comigrate during electrophoresis.

3.7.1 PCR for CT

The *ctxB* PCR confirmed the probe results for all *V. cholerae* strains (Table 28); all CT-probe-positive *V. cholerae*, regardless of serogroup gave the expected amplicon of 460 bp (Figure 16, lanes 6 - 8). The 12 CT-probe-negative, but GM1-ELISA-positive strains and the CT-probe-positive *V. mimicus* (reference strain O115) were negative in the PCR assay for *ctxB*, that is no detectable fragments were amplified. The CT gene sequence for *V. mimicus* must differ sufficiently from those of *V. cholerae* CT to render *V. mimicus* CT undetectable in this PCR.

3.7.2 PCR for TCP

The PCR for *tcpA* exploits the sequence difference between the *tcpA* of the El Tor and classical biotypes of *V. cholerae* O1. It was confirmed that the *tcpA* genes of biotypes El Tor and classical, of the O1 serogroup, were differentiated in the PCR, by amplicon size

(Figure 16, lanes 1 and 2). The *V. cholerae* non-O1 strains could similarly be differentiated in this PCR by type of *tcpA* into two groups (Figure 16, lanes 3 and 4). The fragment amplified was identical to the *tcpA* generated from either biotype El Tor or classical. The apparent doublet band produced by the reference strain O139 (Figure 16, lane 3) may be due to overloading of the gel. The CT-negative strains did not react in the *tcpA* PCR (Table 28). Serogroups O23, O37 and a rough strain possessed a *tcpA* identical to the *tcpA* of classical strains, whereas serogroups O6, O139 and two strains with untypable O antigens possessed a *tcpA* identical to El Tor *tcpA* (Table 28). Only one *ctxB* positive strain, WBDV-101E, belonging to serogroup O49 was negative for *tcpA* by PCR. This strain may either have lost the gene for *tcpA* or it may possess a novel *tcpA* which is sufficiently different from the El Tor and classical *tcpA* to render it undetectable by the primers used in this PCR. The *V. mimicus* (O115) strain was negative for *tcpA*, as it was for *ctxB*, by PCR, indicating that this strain possesses *ctxB* and possibly, *tcpA*, sufficiently different from the *V. cholerae* genes to render the genes undetectable by the primers used in these PCR assays. All TCP positive strains possessed either El Tor or classical type TCP, therefore, CT-positive *V. cholerae* can be divided into two groups by type of TCP regardless of serogroup (Table 28).

3.8 Adhesion

The ability of bacteria to adhere to host tissues is a recognised pathogenic mechanism, and the use of cultured cell lines, such as HEp-2, have been useful for studying bacterial adhesion *in vitro*. Recently, it has been suggested that pathogenic *V. cholerae* non-O1 could be distinguished from non-pathogenic strains by adhesion on the Caco2 cell line (Panigrahi *et al.* 1990). In the present study, both tissue culture cell lines were used to test 4 strains of *V. cholerae* non-O1; ST-positive strain NAG-82E (O14), an environmental strain isolated from cockles E52369 (O?) and two clinical isolates E65313 (O2) and E63807 (O14).

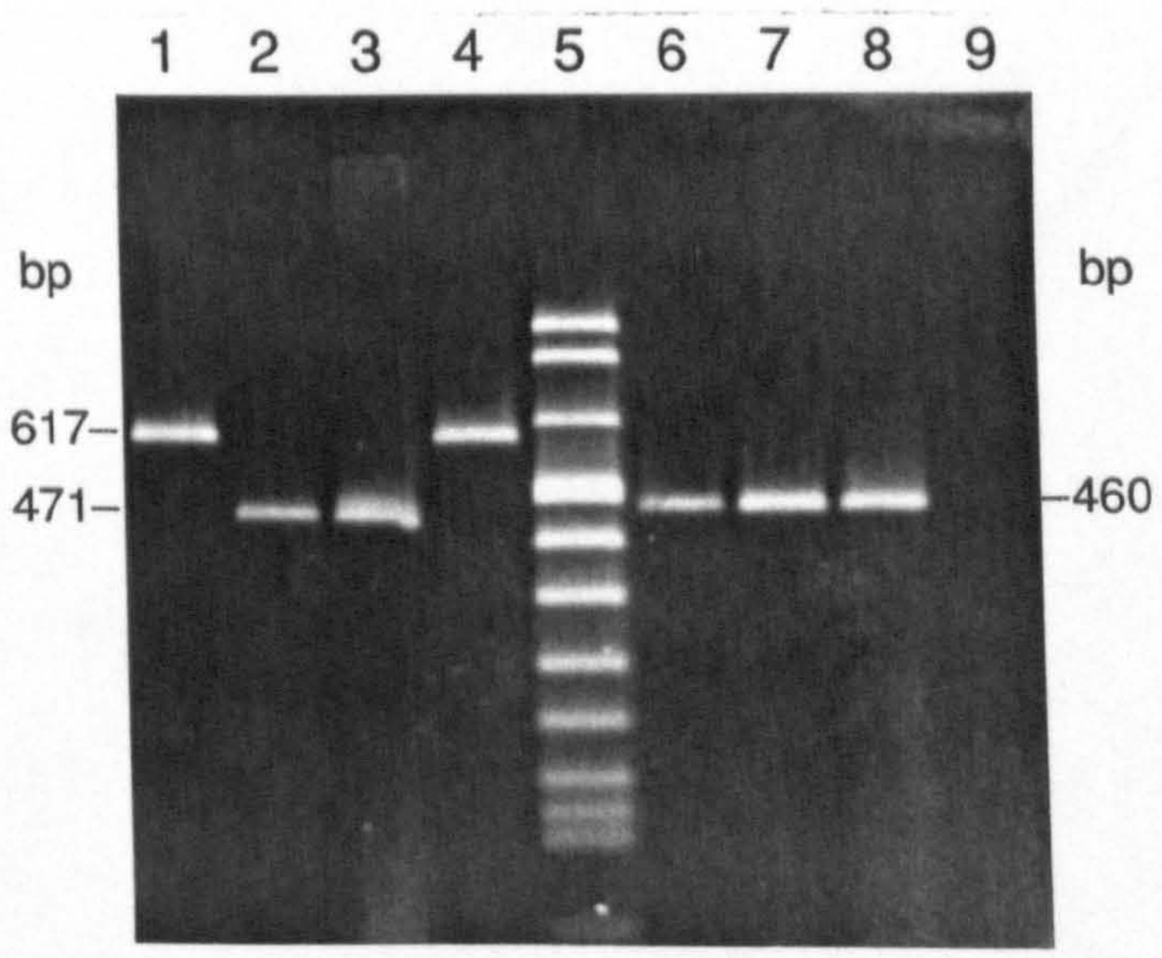
Table 28: Characterisation of *V. cholerae* strains: hybridisation with CT probe and PCR results for *ctxB* and *tcpA*

Strain	Source[a]	Country	Sero-group	Probe CT	PCR *ctxB*	PCR *tcpA*[b]
10954/1	R	?	O1 Ogawa	+	+	E
8457/5	R	?	O1 Inaba	-	-	-
E51116	?	Bangladesh	O1 Ogawa	+	+	C
E46949	D	India	O6	-	-	-
E48065	D	Sri Lanka	O6	-	-	-
E58892	S	Malaysia	O6	-	-	-
N2	W	Australia	O6	+	+	E
N87	W	Australia	O6	+	+	E
317-71	R	?	O23	-	-	-
N7	W	Australia	O23	+	+	C
N9	W	Australia	O23	+	+	C
N92	W	Australia	O23	+	+	C
1322-69	R	?	O37	+	+	C
E54380	D	India	O37	-	-	-
E54381	D	Tunisia	O37	-	-	-
E54418	D	Tunisia	O37	-	-	-
1154-74	R	?	O49	-	-	-
E58864	S	S.E. Asia	O49	-	-	-
WBDV-101E	?	?	O49	+	+	-
E73467	D	India	O49	-	-	-
E85943	D	?	O139	+	+	E
E86270	D	Bangladesh	O139	+	+	E
E87509	D	India	O139	+	+	E
MO45	R	?	O139	+	+	E
E87954	D	India	O139	+	+	E
E88131	D	India	O139	+	+	E
E89007	D	India	O139	+	+	E
E90067	D	Pakistan	O139	+	+	E
E90070	D	Pakistan	O139	+	+	E
E90158	D	?	O139	+	+	E
E90159	D	?	O139	+	+	E
E90251	D	Thailand	O139	+	+	E
E55413	D	Egypt	O?	-	-	-
E55879	D	Egypt	O?	+	+	E
E66371	D	Tunisia	O?	+	+	E
E66824	D	Tunisia	O?	-	-	-
CA385	R	?	O-rough	+	+	C

[a] R = reference strain; W = strain isolated from water; ? = source or country unknown; D = strain isolated from a case of diarrhoea; S = strain isolated from shellfish

[b] *tcpA* PCR results recorded as: C, 617 bp amplicon identical to *tcpA* of the classical biotype; E, 471 bp amplicon identical to *tcpA* of the El Tor biotype; -, negative, no amplicons detected.

Figure 16: Differentiation of *V. cholerae* *tcpA* genes by multiplex PCR and detection of *ctxB* genes.



Lanes 1 - 4 *tcpA* PCR (1) E51116 O1 classical, (2) E51165 O1 El Tor, (3) MO45 O139, (4) 1322-69 O37

Lanes 6 - 8 *ctxB* PCR (6) E51116, (7) MO45 O139 (8) 1322-69 O37

MW markers (bp), 1114, 900, 692, (500, 489), 404, 320, 242, 190, 147, 110, 67.

Negative control, sterile glass distilled water (lane 9).

Suspensions of bacteria (approximately 10^8 bacteria/ml) grown on CFAB agar and strains grown statically in peptone water with and without mannose (overnight, 37 °C) were used. The procedure was performed as described in section (2.3.6a). These strains were also tested for haemagglutination using calf, guinea pig, human and rat rbcs.(2.3.6b). The different growth conditions did not have obvious effects on the results of adhesion tests; however, the presence of mannose did affect the haemagglutination of rbcs. It was found that both the HEp-2 and Caco2 adhesion tests were best performed as 3 h, rather than the conventional 6 h test standardly used for strains of *E. coli*, since *V. cholerae* were found to be cytotoxic for these cell lines. The results are shown in Table 29. All 4 strains showed MS haemagglutination on guinea pig rbcs and MR haemagglutination on human rbcs, 3 of the strains were negative on calf rbcs and MR on rat rbcs, only the NAG-82E strain was negative on rat and MR on calf. All 4 strains were adherent to both cell lines. The adhesion tests were repeated in the presence of sugars other than mannose; no inhibition of adhesion due to mannose, fucose or rhamnose was found.

Forty-four strains of *V. cholerae* non-O1 from both environmental and human sources, including the 4 strains used previously, were grown statically in peptone water and tested in Caco2 and HEp-2 assays and for haemagglutination (in the presence and absence of mannose). All 44 strains adhered avidly to both cell lines except for CA385, the O-rough reference strain (Figure 17). As none of the other strains tested were O-rough strains, there was the possibility that the possession of smooth LPS was necessary for adhesion. To investigate the role of LPS further, 12 wild-type O-rough strains were tested on both cell lines; all 12 were positive, indicating that smooth LPS is not a requirement for adhesion.

In addition to the wild-type strains of *V. cholerae*, the reference strains O2 to O83 were also tested for adhesion on HEp-2 cells only and for haemagglutination. All strains except for those belonging to serogroups O33 (151-68) and O36 (1321-69) were positive for adhesion. The haemagglutination did not appear to correlate with adhesion tests; although the

O-rough reference strain (CA385) was negative for both haemagglutination and adhesion, the O33 and O36 reference strains both gave haemagglutination in the absence of adhesion. Other strains were positive in the adhesion tests but negative for haemagglutination. Due to the avid nature of the adherence observed with *V. cholerae* non-O1 strains, it was difficult to attribute any pattern such as localised, diffuse or aggregative (as assigned for *E. coli* strains). No differences were found between environmental and diarrhoea associated strains. Adhesion was not specific as positive strains attached to both cell lines and glass only; the bacteria would attach to glass coverslips in the absence of tissue culture cells, in a time course experiment bacteria were found to attach to glass only in 5 min, increasing to give full attachment by 90 min.

As it has been postulated that motility (97% of the strains in this study were motile) and, therefore, the possession of flagella might be involved in adhesion of *V. cholerae* to host cells. The role of motility was examined using 23 non-motile strains of *V. cholerae* non-O1 and 1 strain O-rough strain of *V. cholerae*, CA385 (O-rough). One strain of non-motile *V.mimicus* was also tested in HEp-2 adhesion tests. All strains, except *V. cholerae* strain CA385, were positive, indicating that motility is not a requirement for adhesion.

3.8.1 Transmission electron microscopy (TEM).

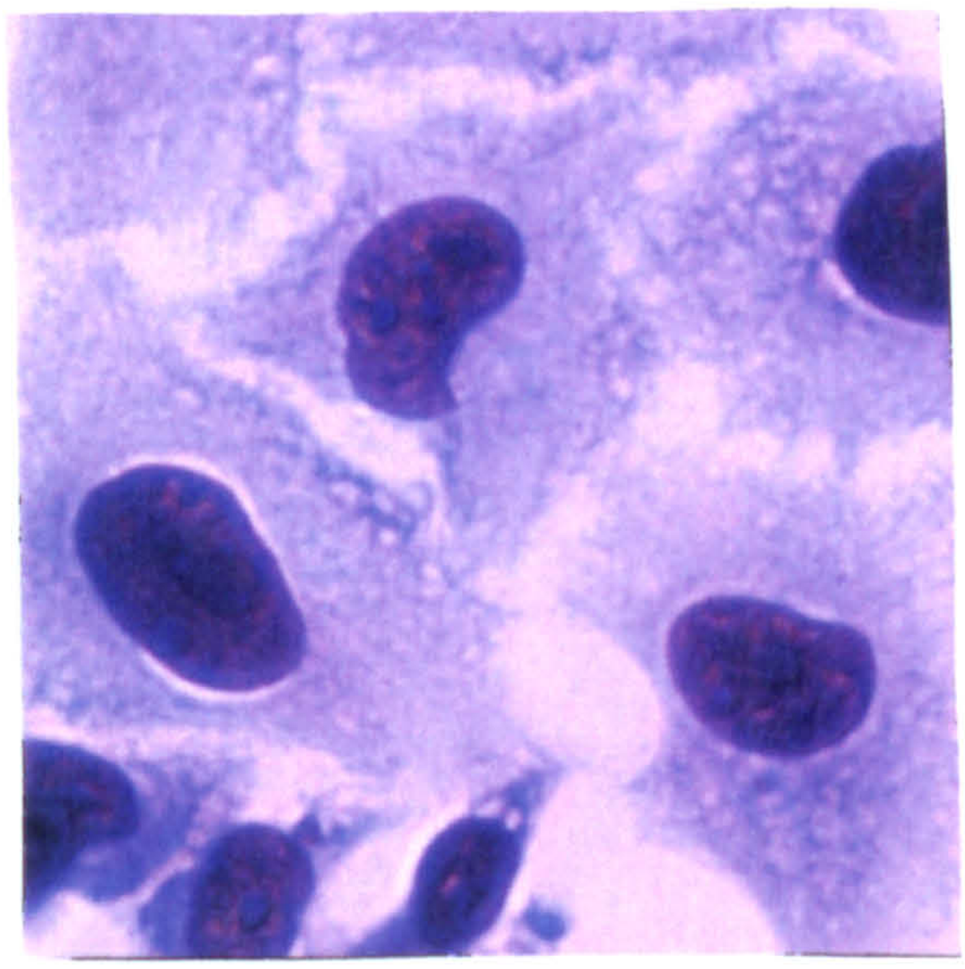
TEM (described in 2.3.6 d) was used to examine 4 *V. cholerae* strains for the presence of pili and flagella. The strains used were a non-adhesive CA385 (O-rough reference strain) and 3 strains positive in the adhesion test, E60544 (O-rough), E55879 (O?) and 334-72 (O26 reference strain). Flagella were not observed on the adhesion-negative, non-motile O-rough strain (CA385); however, flagella were observed on all 3 strains positive in the adhesion test. Pili were not seen on any of the bacteria examined.

Table 29: Adhesion on HEp-2 and Caco2 tissue culture cell lines and haemagglutination of calf, guinea-pig, human and rat red blood cells by *V. cholerae* non-O1.

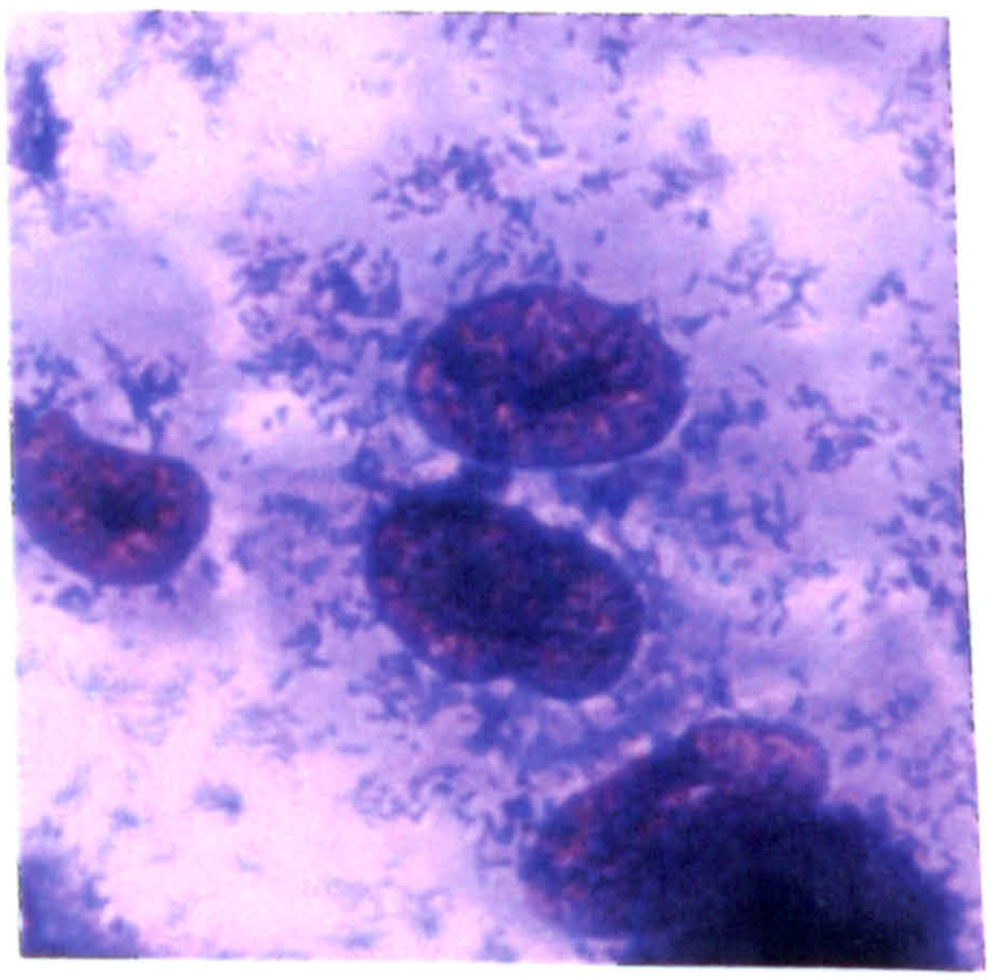
Ref. N°.	Haemagglutination*				HEp-2§	Caco2§
	calf	guinea-pig	human	rat		
NAG-82E	MR	MS	MR	-	+ bacteria on the few cells left, ++ bacteria on glass	Caco2 cells all detached ++ bacteria on glass
E52369	-	MS	MR	MR	+++ bacteria on cells and glass	+++ bacteria on the few cells left and on glass
E65313	-	MS	MR	MR	+++ bacteria on cells and glass	+++ bacteria on the few cells left and on glass
E63807	-	MS	MR	MR	+++ bacteria on cells and glass	++ bacteria on the few cells left and on glass

* MS = mannose sensitive haemagglutination; MR = mannose resistant haemagglutination
§ + = <10 bacteria per cell; ++ = 10 - 20 bacteria per cell; +++ = >20 bacteria per cell

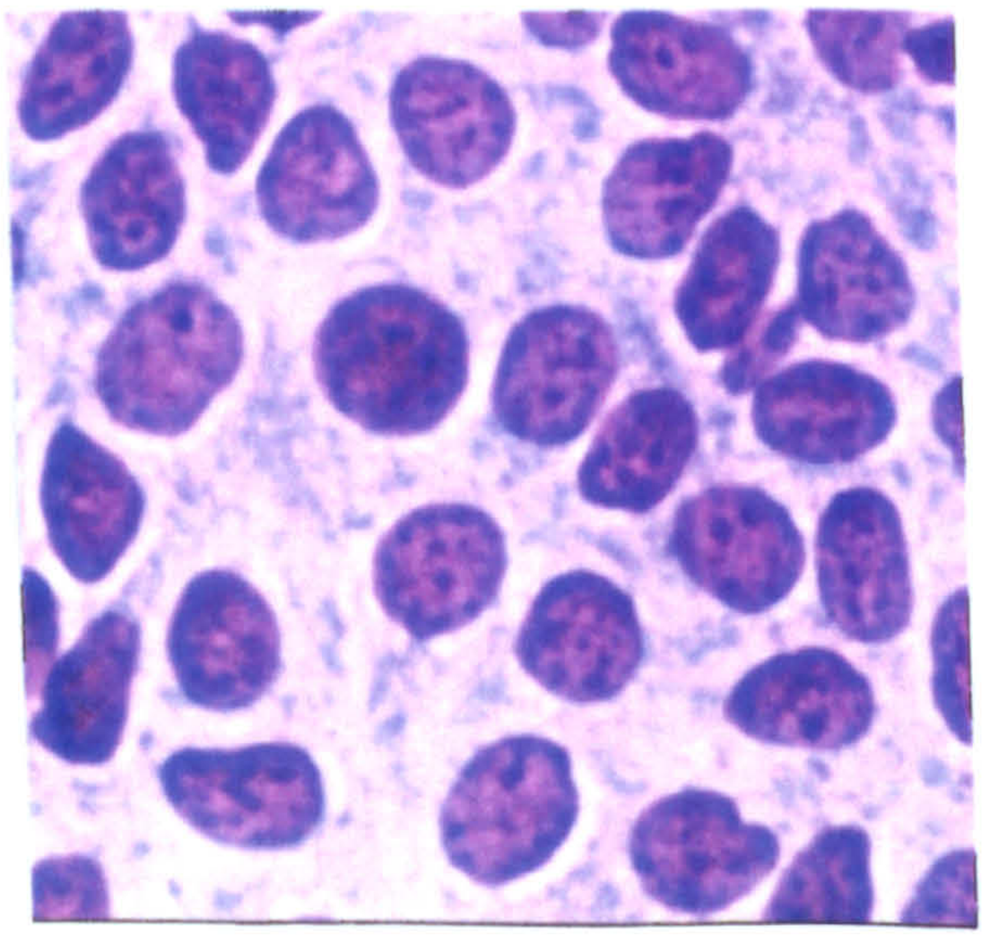
Figure 17: Adhesion of *V. cholerae* non-O1 on HEp-2 and Caco2 cell lines.



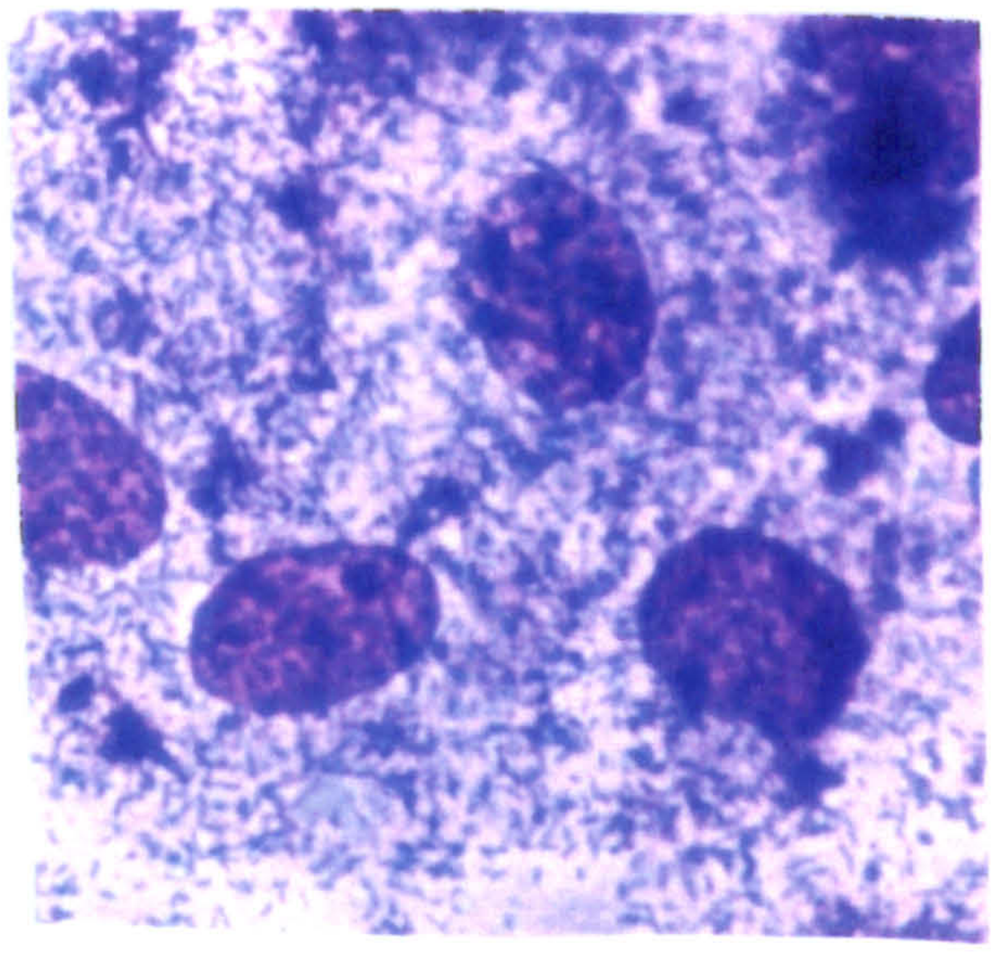
(i) Normal HEp-2 cell monolayer



(ii) Adhesion on HEp-2 cells



(iii) Normal Caco2 cell monolayer



(iv) Adhesion on Caco2 cells

3.9 Plasmid analysis

Although plasmids appear to be uncommon among *V. cholerae*, multiple drug resistance encoded by high molecular weight conjugative plasmids has been reported and in *V. cholerae* non-O1 the carriage of the gene for the thermostable direct haemolysin (*tdh*) is linked to the possession of a large 33 kb plasmid. Plasmid DNA was extracted using a phenol/chloroform extraction method and an alkaline lysis method (described 2.3.5).

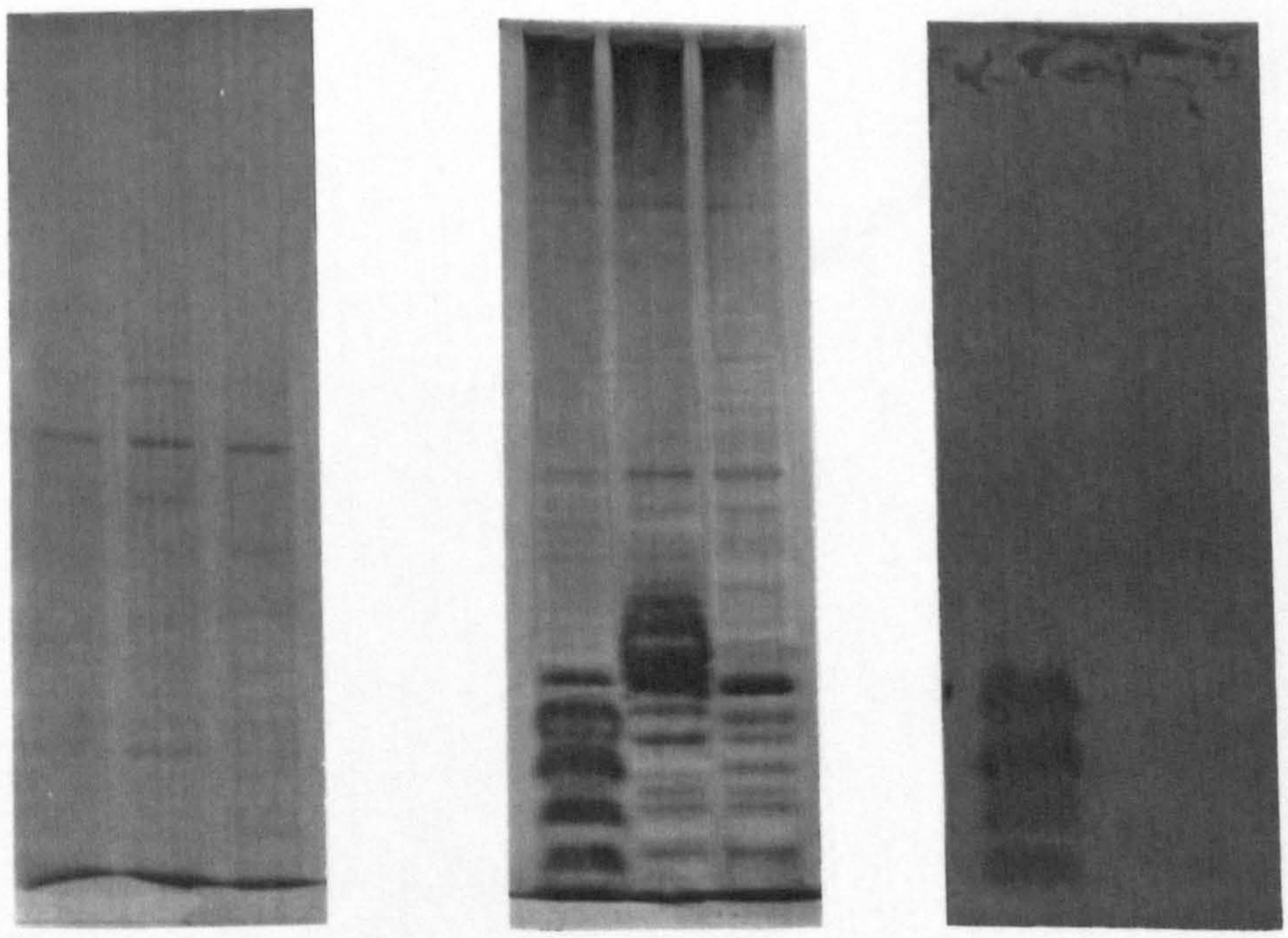
A total of 38 strains, 36 *V. cholerae* non-O1 and 2 *V. mimicus*, were tested using both methods to extract plasmid DNA. As both methods seemed to give the same result the method of Kado & Liu, which was simpler to perform, was preferred. Only 6 of the 38 strains tested possessed plasmid DNA, and three of these were strains isolated from the same patient from a wound (E55414, E55415 and E55416 (O?); two plasmids of 5.0 and 4.6 Mda). The other three strains carrying plasmids were: the CT-probe-positive O37 reference strain (1322-69) carrying a 26 Mda plasmid; a strain belonging to presumptive serogroup 5609 isolated from prawn (E47270) carrying a 4.2 Mda plasmid; and an unidentifiable (O?) clinical isolate from a patient with diarrhoea (E56566) carrying three plasmids of 5.2, 3.7 and 3.0 Mda. All, except the 26 Mda plasmid of strain 1322-69, were small cryptic plasmids (of approximately 5 Mda or less) and all were of unknown significance. The plasmids did not appear to be linked to drug-resistance; the strains E55414-6 and E56566 were sensitive to all drugs tested and 1322-69 and E47270 were both resistant to kanamycin only. Plasmids were not detected in the 2 *V. mimicus* strains tested.

3.10 Protein analysis

3.10.1 Whole cell protein profiles

Whole cell protein profile analysis (described in 2.3.9a) was used to examine strains of serogroup "O26". The O26 serogroup was at one time the largest non-O1 serogroup, with over 40 strains assigned as O26 by serogrouping, but when these strains were examined by

Figure 18: Whole cell protein profiles of *V. cholerae* "O26" strains 334-72, E57025 and E55414, stained for protein, LPS and radio-immunoblotted with antiserum raised against 334-72.



(1) coomassie blue stain for protein

(2) silver stain for LPS

(3) immunoblot

The radio-immunoblot only reacts with the four "paired" bands of 334-72 (O26 reference strain) which are darkly stained with silver stain.

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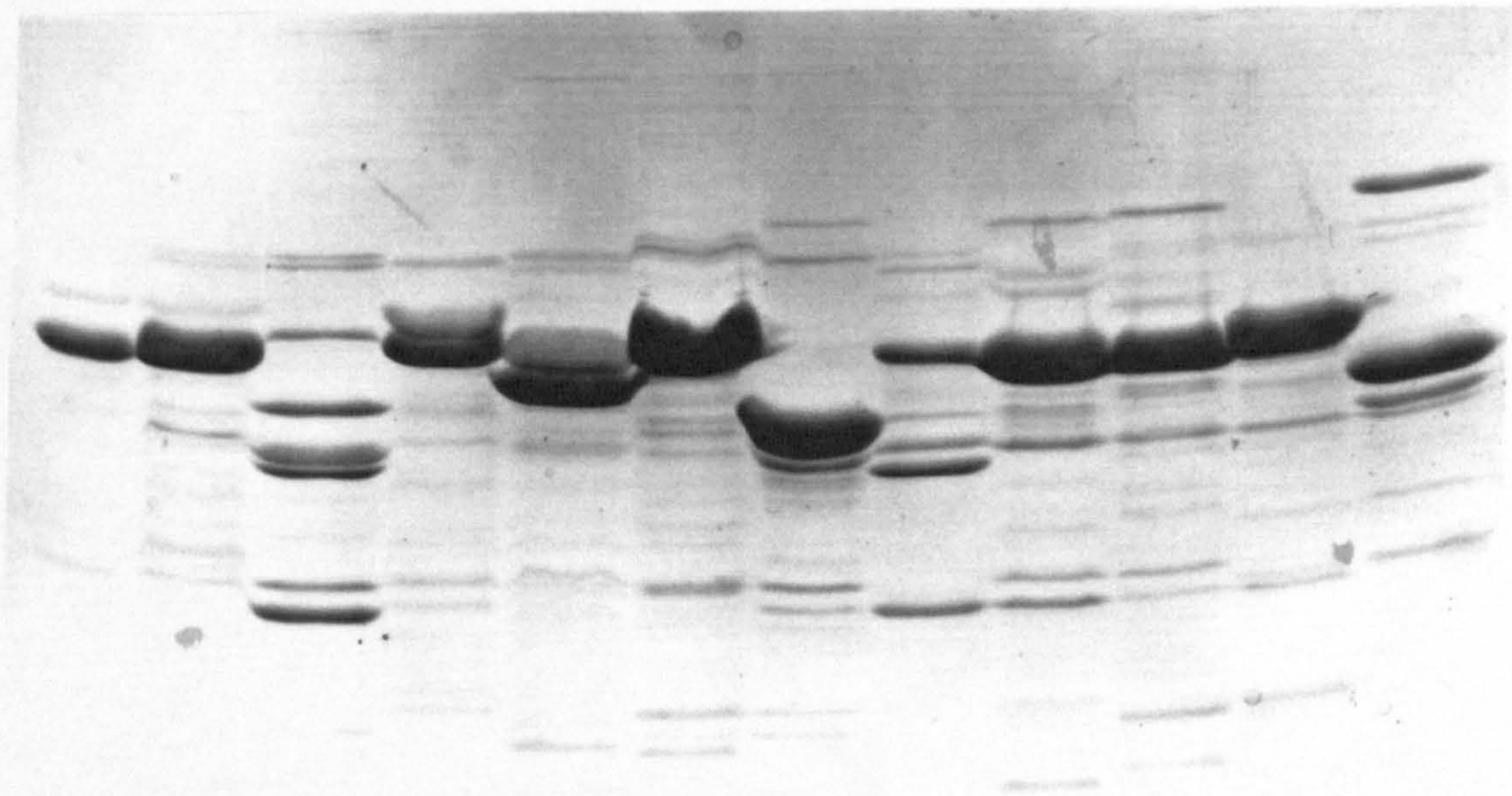
whole cell protein profiles and by LPS analysis (see below) the strains showed marked heterogeneity. *V. cholerae* strains 334-72, E55414 and E57025 were also examined by whole cell protein profile analysis using SDS-polyacrylamide gel electrophoresis. Whole cell profiles were run in triplicate, and a third of the gel was stained with coomassie brilliant blue (for protein bands), a third with silver stain (for LPS) and a third was used for immunoblot analysis. The gel was blotted onto nitrocellulose and reacted with antiserum raised against the O26 reference strain (334-72), after washing, antibody binding was detected using an anti-rabbit IgG immunoglobulin conjugated with radioactive iodine (this procedure was carried out with assistance from Henrik Chart, LEP). As shown in Figure 18, only the 4 paired LPS bands shown by strain 334-72 reacted on immunoblot, even though the other two strains were O26 positive by standard serogrouping methods.

3.10.2 Outer membrane protein (OMP)

The outer membranes of vibrios contain several proteins (OMPs) which are strongly antigenic, *V. cholerae* O1 possess 4 different OMP in the 25 - 45 kda range. Outer membrane proteins were examined for strains of *V. cholerae* non-O1. Outer membranes were isolated from 12 reference strains of *V. cholerae* with O-groups: O-rough (CA385), O1 (E51116), O2 (NCTC 4711), O44 (112-73), O25 (14821-62), O27 (10432-62), O53 (1157-74), O56 (475-75), O62 (1-76), O78 (27-76) and 2 *V. mimicus* strains O20 (317-71) and O71 (162-78). These were examined by SDS-PAGE using 12.5% separation gels (2.3.9b). Among these 12 strains there were 7 different OMP profiles designated by the size of the major OMP (Figure 19). Six strains belonged to profile OMP1, and the other 6 strains belonged to profiles OMP2 - OMP7 (Table 30). The most common major OMP (approximately 38 kda) was found in 8 strains; *V. cholerae* O1, O-rough, O44, O53, O56, O62 and in *V. mimicus* O20 and

Figure 19: Outer membrane protein profiles of *V. cholerae* and *V. mimicus*.



1. CA385 (O-rough)
2. E51116 (O1)
3. NCTC 4711 (O2)
4. 112-73 (O44)
5. 317-71 (O20)
6. 162-78 (O71)
7. 14821-62 (O25)
8. 10432-62 (O27)
9. 1157-74 (O53)
10. 475-75 (O56)
11. 1-76 (O62)
12. 27-76 (O78)

Table 30: Outer membrane protein profiles of *V. cholerae* and *V. mimicus*.

	Ref. N°.	Serogroup	OMP profile
V. cholerae	CA385	O-rough	OMP1 (38 kda)
	E51116	O1	OMP1
	NCTC 4711	O2	OMP6 (no major OMP)
	112-73	O44	OMP2 (40 and 38 kda)
	14821-62	O25	OMP4 (35 kda)
	10432-62	O27	OMP7 (no major OMP)
	1157-74	O53	OMP1
	475-75	O56	OMP1
	1-76	O62	OMP1
	27-76	O78	OMP5 (36 kda)
V. mimicus	317-71	O20	OMP3 (38 and 37 kda)
	162-78	O71	OMP1

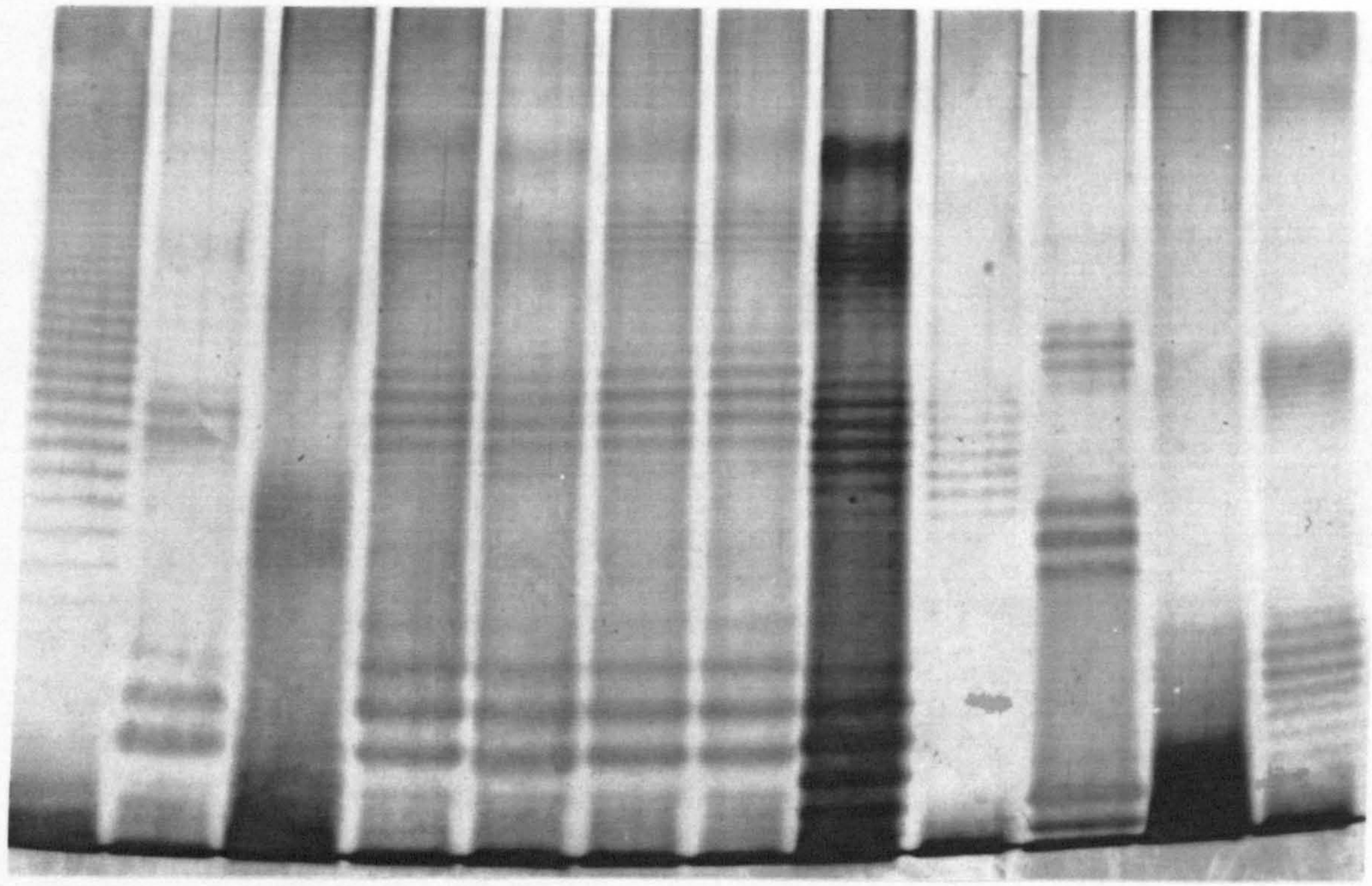
V. mimicus O71. *V. cholerae* O44 and *V. mimicus* O20 differed from the other 6 strains because they both had two major OMP bands, in addition to the 38 kda band. *V. cholerae* O44 had a second major OMP at approximately 40 kda (OMP2) and *V. mimicus* O20 had a second major OMP at approximately 37 kda (OMP3). *V. cholerae* O25 and O78 had 1 OMP each at approximately 35 kda and 36 kda, respectively. The most unusual strains were *V. cholerae* O2 and O27, which did not appear to possess major OMP but were designated different OMP profiles by their minor OMP patterns. There appears to be considerable diversity in OMP of *V. cholerae* and *V. mimicus*, with the majority having a major OMP at around 38 kda.

3.11 Lipopolysaccharide (LPS)

The LPS of *V. cholerae* O1 and O139 are important protective antigens and considered virulence factors. The results presented in the section on serogrouping (3.1.3) indicate the association of certain non-O1 and non-O139 serogroups, such as O2, and O5, with diarrhoeal disease. A sensitive silver staining method was used for detecting LPS in SDS-polyacrylamide gels, as described in 2.3.7.

SDS-PAGE analysis of the lipopolysaccharide of *V. cholerae* non-O1 has proved a useful technique for confirming outbreaks of otherwise untypable strains and for clarifying problems with identification of serogroups, such as O26. Several serogroups showed marked heterogeneity, rather than identity of LPS on silver stained SDS-polyacrylamide gels. On further investigation several new serogroups were identified and antisera raised to these presumptive serogroups (eg: presumptive serogroup 5609, which arose from the O26 investigation). It was also of interest that certain serogroups, in particular O1 and O139, proved particularly difficult to analyse by this technique; instead of the expected sequence of bands a smear was observed, which may possibly due to capsular material. Rough strains also did not give any banding but, the appearance was clear not smeared, with a very darkly staining area at the end the gel lane. In general the LPS of *V. cholerae* and *V. mimicus* was identical

Figure 20: Silver stained SDS-PAGE LPS profiles



Lane 1 *E. coli*, Lane 2 - 12 *V. cholerae* non-O1 strains, all were unidentifiable (O?) except for the four strains in lanes 4 -7 which belonged to serogroup O14.

within serogroups, the patterns obtained had an atypical appearance in that they were irregular, the LPS bands of organisms such as *E. coli* give regular "ladder" structured LPS (Figure 20). Serogroups gave different patterns, the O? strains in Figure 20 were all distinct from each other indicating that they belong to different serogroups. Strains belonging to the same serogroup, such as O14 (Figure 20 lanes 4 to 7) gave identical reproducible, LPS profiles. Serogroups O2, O5, O6, O8, O10, O11, O14 and O37 were tested and all were identical within respective serogroups. In contrast certain serogroups O13, O24, O26 and O69 were heterogeneous and therefore were investigated further (3.11.2).

3.11.1 Analysis of strains which were unidentifiable (O?).

Selected strains which were unidentifiable (O?) with antisera raised against serogroups O1 to O83 and O139 were examined by LPS analysis, to identify any common serogroups. The 2 O? CT probe-positive strains, E55879 and E66371, were shown to have different LPS profiles and therefore belonged to different serogroups. CT probe-negative, but GM1-ELISA positive O? strains were also examined to see if they belonged to common serogroup(s), but the LPS was heterogeneous in all 20 strains tested and no obvious common groups were found.

Strains originating from family outbreaks (section 3.1.3, Table 11) were also examined; by conventional serogrouping different serogroups were identified in 3 "outbreaks" (this was confirmed by LPS analysis), the remaining 2 outbreaks were caused by serogroup O2 (LPS identity was confirmed on SDS-PAGE) and serogroup O?. The 3 O? strains in this outbreak from Malta appeared indistinguishable by conventional serogrouping, however, the R-type result showed that while 2 strains were colomycin and ciprofloxacillin resistant, 1 strain was colomycin and ampicillin resistant. When these three strains were run on an SDS-PAGE and silver-stained, the LPS profiles were identical, indicating that the 3 strains probably belonged to the same serogroup.

The LPS profiles of a group of *V. mimicus* O? strains were also investigated. These 5 strains, 4 from the environment (E40213 from seawater, E58894, E64109 and E64110 from seafood) and 1 clinical isolate (E63453, from a patient with diarrhoea, returned from Thailand) had the same LPS profile, indicating that they belonged to the same serogroup. These same 5 strains were the only *V. mimicus* found to be aerobactin negative (3.12.1).

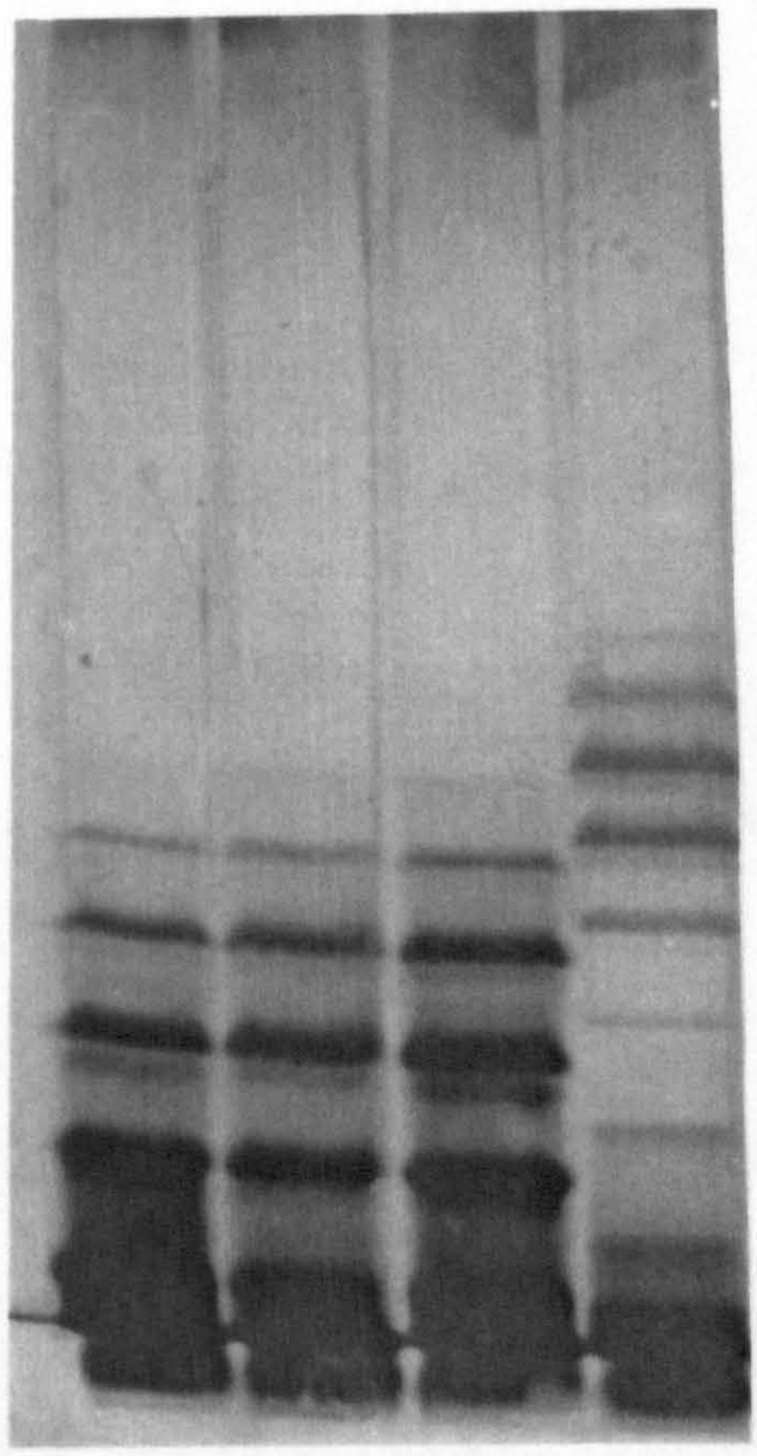
3.11.2 Identification of new serogroups of *V. cholerae* non-O1 using SDS-PAGE analysis.

On further examination of strains belonging to serogroups O13, O24, O26 and O69 it was found that the O13 and O24 serogroups could both be split into 2 distinct groups (Figure 21 shows the 2 profiles found within the "O24" serogoup), and this could be confirmed by conventional serology which showed that the cross-reactions could be absorbed out of the O13 and O24 antisera respectively. The O26 and O69 strains were very heterogeneous groups all different from the reference strain (Figure 23 and 22).

The O69 serogroup was one of the commonest typable O-groups, with isolates from mainly environmental, but also human, sources. There were 20 strains found to be O69 by serology, however, by LPS profile this group was shown to be heterogeneous and none of the 19 wild type strains were identical to the reference strain. Although none of the strains were identical to the reference strain some wild type strains gave LPS profiles identical to each other, these were; (1) E46805 and E46806 both clinical isolates from Thailand, (2) E52349 (isolated from cockles, country of origin unknown) and E72237 (isolated from a river in Chile) and (3) E72241 and E72246 (both from Chilean river water). The LPS profile results were confirmed in conventional serology by absorbing the antiserum with the cross-reacting strains.

As stated in 3.10.1, above, the O26 serogroup was at one time the largest non-O1 serogroup, with over 40 strains assigned as O26 by conventional serogrouping. By LPS analysis there were more than 8 different LPS profiles. The most common profile (15 strains) was identical to E55609, antiserum was raised against E55609 and strains designated as

Figure 21: Silver stained SDS-PAGE LPS profiles of *V. cholerae* "O24"



V. cholerae non-O1 identified as serogroup O24 could be divided into two separate groups by LPS analysis, the two groups had distinct LPS profiles.

(1) 14438-62 (O24 reference strain)

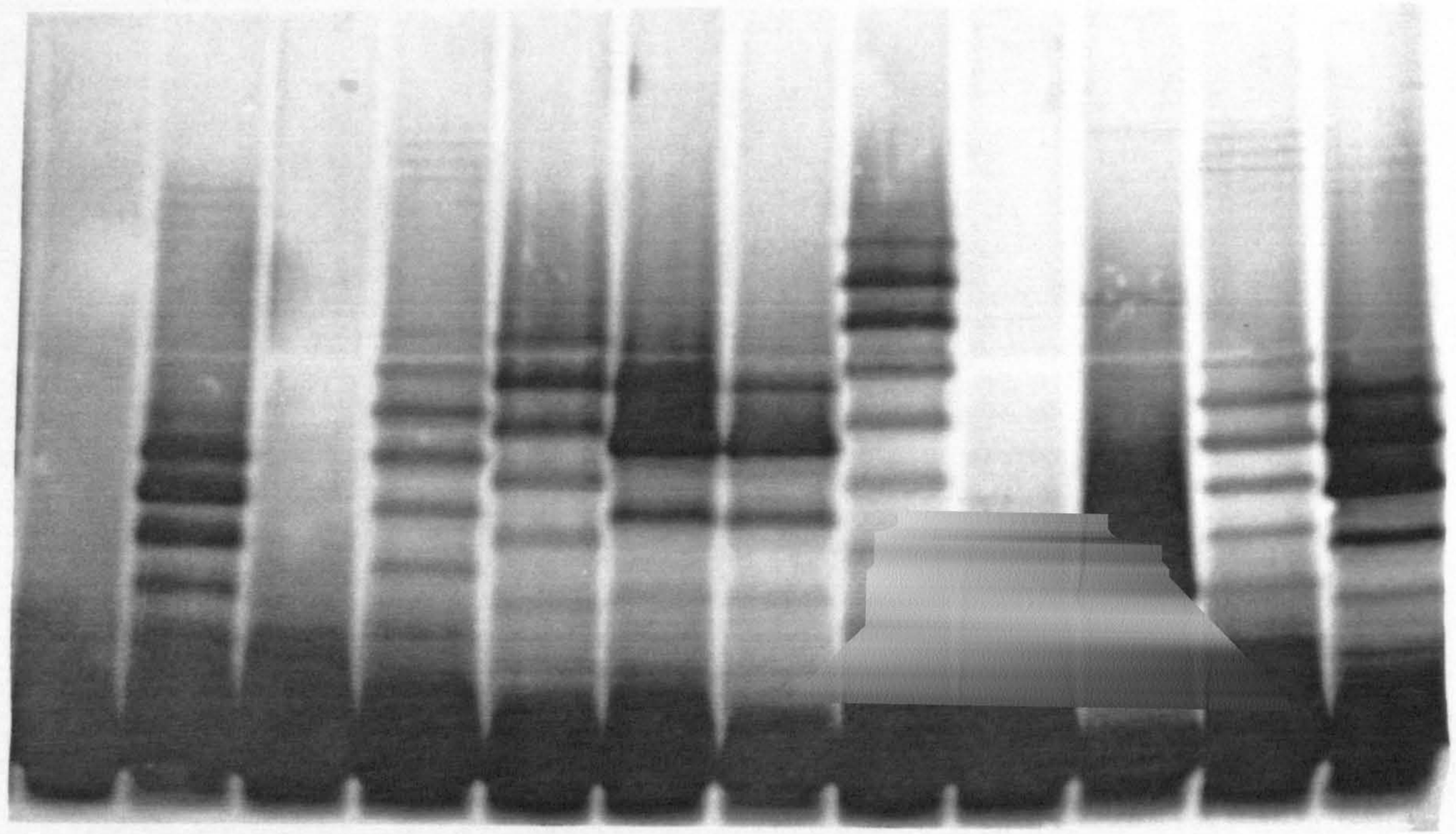
(2) E50053

(3) E54402

(4) E48223

Figure 22: Silver stained SDS-PAGE LPS profiles of

***V. cholerae* "O69"**

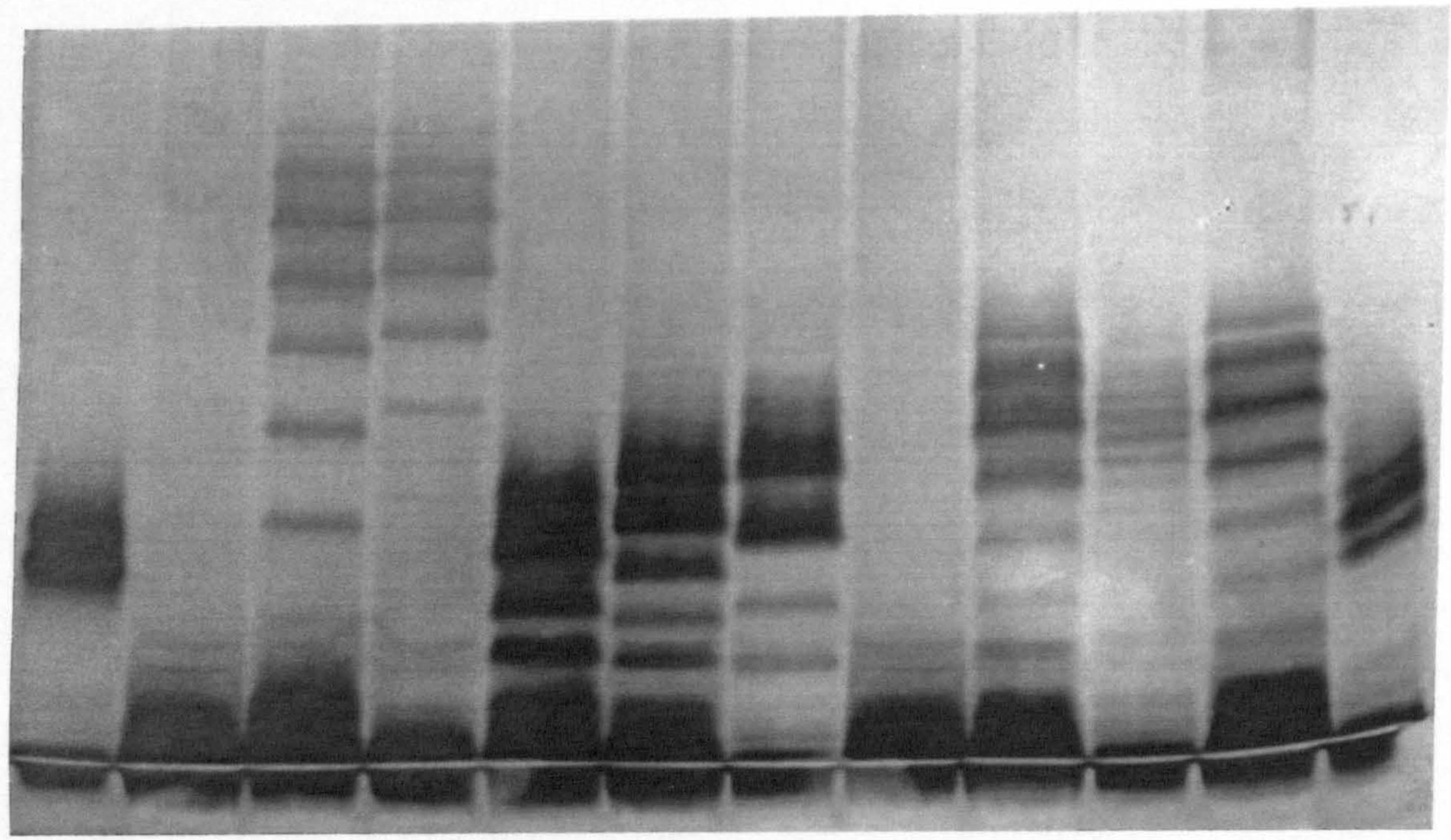


The heterogeneity of the LPS profiles of *V. cholerae* non-O1 strains identified as belonging to serogroup O69.

1. E54922	4. E46815	7. E46805	10. E46007
2. E52349	5. E46814	8. E46251	11. 1861-79 (reference strain)
3. E48641	6. E46806	9. E46238	12. E43433

Figure 23: Silver stained SDS-PAGE LPS profiles of

***V. cholerae* "O26"**



The heterogeneity of LPS profiles from *V. cholerae* non-O1 strains identified as belonging to serogroup O26.

1. E70987	4. E62589	7. E46825	10. E46801
2. E70988	5. E62635	8. E38598	11. E57025
3. E44470	6. E55609	9. E46827	12. E66825

presumptive serogroup 5609, see appendix 2. There was only 1 wild type strain (E81243) which was identical to the O26 reference strain (334-72). The "O26" LPS profile results were confirmed in conventional serology by absorbing the antiserum with the cross-reacting strains. The remaining strains could not be identified and were assigned as O?.

3.12 Production of siderophores

Siderophores are low molecular weight, high affinity iron binding compounds which form part of an inducible iron uptake system expressed by most aerobic bacteria. Although a family of siderophores are recognised, the two major siderophores are the phenolic enterobactin (enterochelin) and the hydroxamate aerobactin. Both siderophores bind to membrane protein receptors; however, aerobactin is recycled. The role of these chelators in bacterial virulence has been well established for certain strains of *E. coli*. However, although strains of *V. cholerae* have been shown to produce a phenolic siderophore, vibriobactin, the role of siderophores in the virulence of strains of *V. cholerae* and *V. mimicus* remains to be elucidated. In the present study strains of *V. cholerae* non-O1 and *V. mimicus* were tested for the presence of these iron chelating compounds using the procedures described in section 2.3.8.

3.12.1 Aerobactin

The ferric perchlorate test was used for the detection of aerobactin (2.3.8b), aerobactin present in culture media binds iron from the ferric perchlorate forming ferric aerobactin which has a distinctive red colour. A total of 82 strains of *V. cholerae* and 50 strains of *V. mimicus* was tested. The *V. cholerae* comprised, 48 *V. cholerae* non-O1 clinical isolates of which 14 were from extraintestinal sites, 34 *V. cholerae* non-O1 from isolated from the environment and one *V. cholerae* O1 (E51116). The 50 strains of *V. mimicus* were all from environmental sources except for 2 strains isolated from patients with diarrhoeal disease. Strains were grown in Tris-succinate medium prior to the ferric perchlorate test.

None of the *V. cholerae* strains was positive for aerobactin, however 45/50 (90%) of the *V. mimicus* produced aerobactin. The 5 aerobactin negative strains were all O? (unidentifiable), but all had the same LPS profile (3.11.1) and therefore belonged to a distinct serogroup.

The ability of bacteria to produce aerobactin has been associated with the ability to produce extra-intestinal infections. To examine this using strains of *V. mimicus*, 6 strains were tested for lethal dose (LD_{50}) in BALB/C mice (conducted under Home Office licence: 70/03323). The strains belonged to serogroups O39 (aerobactin positive strain E64754; aerobactin negative strains E60392 and E64692) and O14 (aerobactin positive strain E45521 and aerobactin negative strains E47169 and E63807). Groups of 5 mice were injected inter-peritoneally with 0.1 mls of bacterial suspension (10^5/ml). The experiment was terminated on day 14, with none of the mice showing any adverse reaction. The production of aerobactin by *V. mimicus* did not appear to be linked to virulence in mice.

3.12.2 Enterochelin

The same strains as used for the aerobactin test were used to examine for the production of enterochelin. Strains of *V. mimicus* did not produce the siderophore enterochelin, including the 5 strains which did not produce aerobactin. Of the *V. cholerae* strains examined, the strain of *V. cholerae* belonging to serogroup O1 produced enterochelin, as did 50/67 (74%) of non-O1 strains isolated from the environment (24/33) and from patients with diarrhoea (25/34). There was no obvious association of enterochelin production with source of isolate or with serogroup. Of the 14 isolates from patients with extraintestinal disease only 3 (21%) produced enterochelin. From this limited study, only strains of *V. mimicus* produced aerobactin, and enterochelin was produced only by strains of *V. cholerae.* Therefore, these tests are discriminatory between strains of *V. mimicus* and *V. cholerae*.

DISCUSSION

4.1 Identification and typing of *V. cholerae* and *V. mimicus*.

Over 1000 strains of *V. cholerae* serogroup non-O1 and *V. mimicus* were used in this study. These strains were isolated from a range of sources and from 69 different countries. The present study found that diarrhoeal disease caused by these organisms in the UK was not an indigenous problem but associated with travellers returning from abroad. In particular, diarrhoeal disease was associated with travel to India, Kenya, Tunisia or Thailand. The environmental strains of *V. cholerae* non-O1 and *V. mimicus*, used in this study, were principally isolated from crustacean shellfish, especially shrimps and prawns, which originated from abroad. These initial findings reflect those of previous studies; both *V. cholerae* and *V. mimicus* are found to be naturally occurring in aquatic environments, widely distributed throughout the world and most cases of diarrhoea occurring in the UK result from travellers returning from areas, such as Africa and Asia, where cholera and cholera-like diseases are prevalent (Janda *et al.* 1988; West 1989; Morris 1990). Cases of diarrhoea caused by *V. cholerae* non-O1 and *V. mimicus* have been associated with a history of consumption of seafood, especially oysters and crustaceans (Morris *et al.* 1981; Wilson *et al.* 1981). There was not an indigenous problem in the UK, despite the presence of *V. cholerae* non-O1 in the environment (Lee *et al.* 1982). This may be due to a number of reasons, including, differences in cooking and eating habits and under-reporting due to these organisms not being suspected in patients who have no history of travel abroad. It may also be that though the organisms are present in the UK, the environmental conditions, such as water temperature, may not favour proliferation. Previous studies have shown that the colonisation of crustacean shellfish, such as crabs, shrimps and lobsters depends on the concentration of bacteria and is observed more

frequently at higher ambient temperatures (Davis and Sizemore 1982; Huq *et al.* 1986).

Fourteen strains of *V. cholerae* non-O1, which were included in the present study, were from cases of extraintestinal disease. More than half of these patients with extraintestinal illness had a known history of travel abroad. These extraintestinal strains were not found to produce CT or heat-stable toxin (NAG-ST). There were no strains of *V. cholerae* belonging to serogroup O1 or *V. mimicus* isolated from extraintestinal sites in this study, however, previous studies have reported the isolation of *V. cholerae* O1 and *V. mimicus* from extraintestinal sites. These studies have also found that where extraintestinal infections have occurred, the strains of *V. cholerae* and *V. mimicus* did not produce CT, indicating the involvement of other pathogenic factors (Johnston *et al.* 1983; Safrin *et al.* 1988; Clark *et al.* 1989). The factors possessed by strains of *V. cholerae* and *V. mimicus* causing extraintestinal infections are still to be elucidated; it may be that these organisms are acting as opportunistic pathogens and that host susceptibility plays a major role (Safrin *et al.* 1988).

All the strains used in the present study were identified biochemically as *V. cholerae* and *V. mimicus*, and characterised by serogroup and biotype, before representative strains were selected for further detailed study of virulence attributes. Strains of *V. cholerae* and *V. mimicus* were distinguished from other vibrios using the biochemical tests described (section 2.2.1). Biochemical differences within *V. cholerae* and *V. mimicus* were very limited and they were differentiated primarily by VP (Vogues-Proskauer) and sucrose fermentation. Within *V. cholerae* the different serogroups could not be distinguished biochemically, although, all strains of *V. cholerae* serogroup O1 fermented mannose and this reaction was variable with the non-O1 serogroups.

Neither biotyping nor phage typing, which have been used to differentiate *V. cholerae* O1 (Furniss *et al.* 1978), were found to be useful in differentiating *V. cholerae* non-O1 and *V. mimicus*. Both *V. cholerae* non-O1 and *V. mimicus* were, with respect to haemolysin production on horse rbc, polymixin sensitivity and for *V. cholerae* non-O1 the VP reaction (all

strains of *V. mimicus*, were by definition, VP-negative), "El Tor-like". Only 4.4% of *V. cholerae* non-O1 reacted in the phage-tying scheme, and the phage typing results were inconsistent for serogroups other than O1. In a previous study, 14.3% of *V. cholerae* non-O1 reacted with the phages (Frost and Rowe 1987), however, only 56 strains were tested, compared to the 500 *V. cholerae* non-O1 tested in the present study.

4.1.1 Serogrouping and association of serogroup with virulence factors.

The single serogrouping scheme based on the O-antigen (Sakazaki and Donovan 1984) was found to apply to both *V. cholerae* and *V. mimicus* strains. Other vibrio species (data not presented) did not react with the antisera raised to reference strains in this study, and therefore could not be serogrouped within the scheme. The majority (56%) of *V. cholerae* non-O1 and *V. mimicus* strains tested were unidentifiable (O?) using antisera raised against O1 to O83 and O139. An extended serogrouping scheme, incorporating 141 serogroups (O1 - O140) has recently been published and this should reduce the number of unidentifiable strains (Shimada *et al.* 1994). Of the identifiable serogroups, O1 and O139 were found to be exclusive to the species *V. cholerae*. The serogroups expressed by *V. mimicus*, were also found in *V. cholerae*. The identifiable *V. cholerae* non-O1 and *V. mimicus* strains (30%) were differentiated into 59 distinct serogroups using 83 antisera raised against serogroups O2 - O83 and O139. Seventy percent of these identifiable strains belonged to only 20 serogroups. Some serogroups, O2, O5, O9, O11, O13, O24, O34, O37 and O139, were associated with clinical isolates from diarrhoeal disease and serogroup O19 was commonly isolated from the environment. Representatives of most serogroups were found from both clinical and environmental sources. The most common serogroups of *V. cholerae* associated with diarrhoeal disease, excluding O1 and O139, were serogroups O2 and O9, both serogroups were also associated with family outbreaks. Strains belonging to serogroups O2 and O9, although closely associated with diarrhoeal disease did not possess toxin genes for CT or NAG-ST, indicating

these strains possessed unknown factors which caused diarrhoeal disease. The present study suggests that the somatic antigen could be a useful indicator of diarrhoeagenic potential within *V.cholerae* non-O1. Other studies have previously suggested the prevalence of the O2, O5, O7 and O37 serogroups in gastrointestinal disease and O4 and O8 in the environment (WHO Scientific Working Group 1980; Donovan 1984). In a study conducted in the USA, strains of *V. cholerae* belonging to serogroup O2, accounted for 43% of the total number of strains of *V. cholerae* isolated (Morris *et al.* 1981). Outbreaks attributable to strains of *V. cholerae* non-O1, have retrospectively, been linked to serogroups O5 and O37 (Donovan 1984); a food-borne outbreak in Czechoslovakia was caused by *V. cholerae* O5 (Aldova *et al.* 1968) and a water-borne outbreak was caused by a CT-producing *V. cholerae* O37 (Kamal 1971).

The O1 and O139 serogroups of *V. cholerae* produce CT, the main virulence factor involved causing the dramatic symptoms of epidemic cholera. *V. cholerae* non-O1 and *V. mimicus* also cause sporadic cases and occasionally outbreaks of diarrhoeal disease, which may be indistinguishable from cholera (Blake *et al.* 1980), however, as shown in this study they rarely produce CT. Therefore, other pathogenic factors are probably involved and these factors may also have associations with serogroup. The correlation of NAG-ST with serogroup and with *V. mimicus* has been suggested (Pal *et al.* 1992). In the present study a correlation was found between serogroup O14 of *V. cholerae* and NAG-ST. In addition the species *V. mimicus*, regardless of serogroup, was associated with two possible virulence factors; the possession of the NAG-ST gene and the production of aerobactin, a powerful iron chelator.

4.1.2 R-tying and plasmid carriage

Strains of *V. cholerae* non-O1 and *V. mimicus* were examined for resistance to antibacterial agents, and for plasmid carriage. The presence of plasmids has often been linked to both resistance and to the acquisition of virulence factors in other organisms.

Strains of *V. cholerae* O139 were consistently found to be resistant to

CoSSuTm (colomycin, streptomycin, sulphathiazole and trimethoprim), the same distinctive R-type pattern was found in four other strains, all of which were unidentifiable (O?) strains isolated from patients who had recently returned from India. The same R-type pattern was not found in other *V. cholerae* or *V. mimicus* or from other parts of the world. CT-positive strains, belonging serogroups other than O139, were also used in this study, but none were found to be multiply resistant to antibiotics. There was no apparent correlation of R-type pattern with other serogroups. The most common resistance of *V. cholerae* non-O1 and *V. mimicus* was to Co (80% of strains tested) and the most common R-type pattern was a single resistance to Co (56%).

The multiple resistance of the *V. cholerae* O139 serogroup is interesting, because it is so distinctive. It is possible that the multiple drug resistance and the CT gene were somehow acquired to "create" this novel serogroup, which did not appear to be present before 1992. In the present study, all unidentifiable (O?) *V. cholerae* non-O1 strains, received in the LEP since 1985, were retrospectively examined with antisera raised against O139 and none reacted (Cheasty *et al.* 1993), indicating that this serogroup had not been found before. Indeed, it has been suggested that the O139 serogroup is derived from a *V. cholerae* O1 biotype El Tor, this is supported by, possession of *ctx* and *tcpA* genes, patterns in multilocus enzyme electrophoresis, ribotyping and pulse field gel electrophoresis (Cravioto *et al.* 1994; Hall *et al.* 1993; Calia *et al.* 1994; Berche *et al.* 1993; Albert 1994). A recent study suggested probable progenitor strains for O139, which were O1 but neither truly classical nor truly El Tor in their biotype attributes (Pajni *et al.* 1995). The O139 strains do appear to be related to O1 El Tor, however, there are differences which indicate that they are not derived from O1, these include; the presence of capsule, the absence of O1 biosynthetic genes, the lack of reaction with the El Tor phage and with antiserum raised against the O1 antigen (Johnson *et al.* 1994; Albert 1994). The high attack rate in adults, indicating the ineffectiveness of pre-existing immunity from O1, also suggests that O139 is a novel strain (Mandal 1993). The results obtained in the

present study using serogrouping, Southern blot analysis of CT genes and PCR of *ctxB* and *tcpA*, suggest that O139 is a novel non-O1 serogroup.

Multiple antibiotic resistance is often linked to plasmid carriage. High molecular weight, multiple-antibiotic resistance plasmids are occasionally found in *V. cholerae* O1. Multiple resistance, encoded by a 100 Mda Inc C conjugative plasmid, has been found in *V. cholerae* O1 outbreaks (Threlfall *et al.* 1980; Threlfall and Rowe 1982) and recently the emergence of multiresistant strains of *V. cholerae* O1 has also been reported from Ecuador (Threlfall *et al.* 1993). However, the multiple resistance of *V. cholerae* O139 strains does not appear to be linked to plasmid carriage, as the examination of O139 strains revealed that only the reference strain (MO45) carried plasmids (personal communication Dr. J. Threlfall). Other *V. cholerae*, non-O1 and non-O139, and *V. mimicus* were examined for plasmid carriage in the present study (section 3.9). *V. mimicus* did not carry plasmids and only 15% of *V. cholerae* strains carried small cryptic plasmids of approximately 5 Mda or less. Only one *V. cholerae* non-O1 strain carried a larger plasmid, this was the CT-probe-positive O37 reference strain (1322-69) which carried a 26 Mda plasmid, of unknown significance. Plasmid carriage in these strains did not seem to be linked to drug-resistance nor to pathogenic factors. Previous studies have also found that plasmid carriage is infrequent among *V. cholerae* and most plasmids are cryptic and of low molecular weight (Janda *et al.* 1988). Attempts to correlate extrachromosomal elements with phenotypic or virulence characteristics have generally been unsuccessful (Newland *et al.* 1984). Although the thermostable direct haemolysin (NAG-rTDH) is plasmid-mediated in *V. cholerae* non-O1 (Honda *et al.* 1986). The *tdh* gene is encoded on a large 33 kb plasmid in *V. cholerae* and probably originated from the chromosome of *V. parahaemolyticus* (Nishibuchi *et al.* 1985; Nishibuchi and Kaper 1985). The strains in the present study were not examined for TDH, however, of the strains which were examined for plasmid carriage none possessed a large 33 kb plasmid.

4.2 Pathogenic mechanisms and virulence

Pathogenicity is the ability to produce disease, and pathogenic bacteria may show variation in pathogenicity relating to the virulence of a given organism. A range of symptoms has been associated with cases of *V. cholerae* non-O1 gastroenteritis, including fever and bloody diarrhoea which are encountered only rarely in "typical" cases of cholera. The wider range of presenting symptoms seen with *V. mimicus* and *V. cholerae* non-O1 as opposed to *V. cholerae* O1 infections is probably related to the presence or absence of a number of virulence factors.

4.2.1 Cholera Toxin (CT)

The characteristic symptoms of clinical cholera result from the action, on the gut mucosa, of a single subunit protein toxin, CT. In this study tissue culture cell assays, specific DNA probes and immunoassays were used to detect CT.

The CT genes of *V. cholerae* and *V. mimicus* were detected using polynucleotide probes CTA and CTB and an oligonucleotide probe GM1. The possession of the genes for CT correlated with the somatic antigens expressed; 87% of *V. cholerae* O1 and 100% of *V. cholerae* O139 carried the CT genes. In contrast, < 1% of *V. cholerae* non-O1, non-O139 and *V. mimicus* hybridised with the probes. All CT positive strains possessed the genes for both A and B subunits. Previous studies have reported CT production in strains of *V. cholerae* non-O1 from Bangladesh and India; Datta-Roy (Datta-Roy *et al.* 1986) found an incidence of 26% (9/34) in clinical isolates and 10% (1/10) in environmental isolates. The incidence in other parts of the world is lower. A study in Thailand (Hanchalay *et al.* 1985) found none of 44 human isolates and 2% (5/237) of environmental isolates were CT positive. In a study of environmental isolates from the Louisiana Gulf coast (Roberts *et al.* 1982) it was found that only 0.3% (7/2500) produced CT. The present study also found a low incidence of CT-positive strains (0.25%) in *V. cholerae* non-O1, non-O139 (the incidence increases to 2% if

O139 strains are included); all CT-positive strains of *V. cholerae* non-O1 were clinical isolates from cases of diarrhoea and no CT-positive strains were found from environmental sources.

A polymerase chain reaction was also used to detect the *ctxB* gene in CT-positive strains and to look for *ctx*-related genes in other *V. cholerae* non-O1, in particular the CT-probe-negative, but GM1-ELISA positive strains (Said *et al.* 1994; Said *et al.* 1995). The putative toxin produced by these strains may share epitopes with CTB and related gene sequences. All CT-probe-negative strains were negative by PCR for *ctxB* and therefore did not possess related gene sequences. CT-positive *V.cholerae* non-O1 *ctxB* was identical to the *ctxB* of *V. cholerae* O1 strains. A multiplex polymerase chain reaction (PCR) directed against CT gene, *ctxA*, and the TcpA gene, *tcpA*, has been used to differentiate biotypes of *V. cholerae* O1 (Keasler and Hall 1993). The CT-positive *V. cholerae* non-O1 could be similarly differentiated by *tcpA* PCR (Said *et al.* 1995).

The production of CT was demonstrated directly using immunoassays, VET-RPLA and ELISA, for all CT-probe positive strains. However, the use of tissue culture assays for the detection of CT was limited to *V. cholerae* O1, the reference strain O-rough (CA385), which was originally derived from an O1 strain, and the *V. cholerae* O139. Other *V. cholerae* non-O1, which were CT-positive, produced a cytotoxin which masked CT production. Even when different culture media and different growth conditions were used to optimise conditions for the production of CT, the masking effect of the cytotoxin could not be overcome.

4.2.1a The masking of CT by cytotoxin

The production of CT in O1 (E51116) and O37 (1322-69) as detected by VET-RPLA (section 3.3.3a) gave identical titres. CT and cytotoxin titres were compared in Y1 cell assay, both toxins were heat-labile and first detected soon after the beginning of the log phase of growth. However, the CT titre levelled out earlier than the cytotoxin titre, therefore, the cytotoxic titre was always greater and able to mask the CT titre in *V. cholerae* O37. Both CT

and cytotoxin were exported out of the periplasm (3.3.3b), but CT was present intracellularly whereas no intracellular cytotoxin was found, this difference may be due to the way in which CT and cytotoxin are exported. The titres of both toxins were also found to decrease after the end of the log phase of growth, at this stage the organisms probably stop actively producing toxins and the exported toxins begin to break down due to the presence of proteases and other enzymes.

The cytotoxin produced by *V. cholerae* O139 differed from the cytotoxin of other *V. cholerae* non-O1 examined, in that it did not mask the CT effect. It may be that *V. cholerae* O139 are producing much larger amounts of CT, or conversely lower amounts of cytotoxin, than other *V. cholerae* non-O1.

4.2.1b Southern blot analysis of the CT gene.

DNA preparations were made of CT-probe-positive strains and the chromosomal DNA digested with *Hin*dIII. Southern blots were prepared and hybridised with CTA+B polynucleotides or GM1 oligonucleotide probes. The CT-positive *V. cholerae* strains could be differentiated into 7 different patterns by size of hybridising fragment. An eighth pattern belonged to the only CT-positive *V. mimicus* (reference strain 523-80, serogroup O115) found in the present study. A single hybridising band of 22 Kb was found in 6 CT-positive *V. cholerae* non-O1, belonging to serogroups O6, O49 and O105. This band appeared to be similar or identical to the single band of *V. cholerae* O1 El Tor (10954/1). Strains belonging to serogroups O139 and O23 had 2 hybridising fragments and *V. cholerae* O1 classical (E51116) had 3 fragments indicating the presence of more than one CT gene in these strains.

The DNA preparations of *V. cholerae* O139 were also digested with *Bgl*II and *Xba*I. When *Xba*I and *Bgl*II were used in combination as recommended by Das *et al* (Das *et al.* 1993), the result was a complex series of bands ranging from 7 kb to > 25 kb. The fainter, higher molecular weight were thought to be partial digest products; there were two main

hybridising fragments (7 and 7.8 kb), confirming the *Hin*dIII digest result of at least 2 gene copies. The *V. cholerae* O139 were not all identical, and this variation in the size of hybridising fragment indicated that serogroup O139 strains are not strictly clonal in origin. However, there was no evidence to suggest that specific clones are associated with certain geographical areas.

Previous studies have shown that the CT operon is situated in the *V. cholerae* chromosome and may be present in multiple copies in strains of the classical biotype, but in a single copy in most strains (70%) of the El Tor biotype (Moseley and Falkow 1980; Kaper and Levine 1981). Another study hybridised *Hin*dIII digests with a CTA probe and found that classical biotype possessed 2 hybridising fragments, the El Tor biotype from eastern countries 1 fragment and the El Tor biotype from the USA 2 fragments (Cook *et al.* 1984). Similarly Australian *V. cholerae* O1 were found to be genetically diverse and unrelated to strains from Singapore, Jakarta and Louisiana (Desmarchelier and Senn 1989). A survey in Louisiana found that *V. cholerae* non-O1 and *V. mimicus* which hybridised with the CT probe, showed greater genetic diversity than the highly conserved USA *V. cholerae* O1 strains (Kaper *et al.* 1986). Based on the toxin gene RFLP and total chromosomal restriction patterns at least two separate clusters of *V. cholerae* non-O1 were distinguished in the Louisiana study, in addition to a distinct cluster of *V. mimicus* strains. The present study demonstrated at least 3 distinct clusters of *V. cholerae* non-O1 and an additional *V. mimicus* pattern. The CT of *V. cholerae* non-O1 and *V. mimicus* is located on the chromosome usually as a single gene copy but in certain serogroups, O23 and O139 as multiple copies. Duplication of the CT genes in the classical biotype and 2 or more *ctx* copies present on tandemly repeated genetic elements (7kb or 9.7kb) in some strains of the El Tor biotype have been reported (Mekalanos 1983). Similarly tandemly repeated *ctx* copies have been reported to occur in *V. cholerae* O139 (Das *et al.* 1993).

4.2.2 Production of a putative toxin by strains of *V. cholerae* non-O1 and *V. mimicus*, as detected by GM1-ELISA.

Strains of *V. cholerae* which do not produce CT have been isolated from patients with diarrhoeal disease (McIntyre *et al.* 1965; Morris *et al.* 1984; Batchelor and Wignall 1988) and a volunteer study has shown that *V. cholerae* non-O1 which do not possess the CT gene can elicit diarrhoea with a severity comparable to cholera (Morris *et al.* 1981). These so-called non-toxigenic strains may be producing a toxin distinct from CT and evidence has been found of the production, by an environmental *V. cholerae*, of a second enterotoxin, which was different from CT in antigenic nature, receptor site, mode of action and genetic homology (Saha and Sanyal 1988).

Of the *V. cholerae* non-O1 and *V. mimicus* strains, which did not possess the gene for CT, 19% were, nevertheless, found to be positive in the GM1-ELISA assay. A positive result was obtained with antisera against CT and CTB, but a not with an antiserum prepared against LT. These strains were, therefore, suspected of producing a putative toxin which had the ability to bind the ganglioside GM1 and shared antibody binding sites with CT, and more specifically with CTB. The B subunit of CT is responsible for specific high affinity binding of the toxin to the GM1 gangliosides of intestinal epithelial cells (van Heyningen *et al.* 1974; Holmgren *et al.* 1973; Svennerholm and Holmgren 1978). Therefore, the strains expressing the putative toxin were tested with a specific GM1 oligonucleotide probe, but none of the strains hybridised with this probe. When strains were tested in a double sandwich ELISA, in which GM1 was not used, the most successful ELISA used anti-LT as the first ligand and anti-CTB as the second ligand, even though anti-LT gave a negative test when used in a GM1-ELISA. Interestingly, the two ELISA tests which used anti-CTB as the first ligand were totally negative, the reasons for these differences are not known but may be due to the conformation of the protein, which could be effected by the different antisera.

The GM1-ELISA positive strains were also tested with the CTA+B combined

polynucleotide probe at low stringency, which allowed detection of LT and CT gene sequences. CT and the LT of *E. coli* are part of a heterogeneous family of enterotoxins and different forms of LT have been described ie: LTh-I, LTp-I, LTIIa and LTIIb (Guth *et al.* 1986). Similarly different forms of CT may exist with differences in molecular structure between enterotoxins produced by different serogroups of *V. cholerae* (Finkelstein *et al.* 1987). A previous study has demonstrated that toxin purified from a *V. cholerae* non-O1 from Louisiana was indistinguishable from cholera toxin and that another toxin purified from a *V. cholerae* non-O1, from a patient with diarrhoea, was biologically and immunologically similar but not identical to CT in molecular structure (Yamamoto *et al.* 1983b). However, none of the strains producing the putative toxin in the GM1-ELISA hybridised with the CTA+B probe at low stringency, this may be because there is insufficient homology in the gene sequences of the putative toxin and CT, despite both being detected by the GM1-ELISA.

Attempts were made to provide further evidence for the existence of the putative toxin using other immunoassays, such as PIH, colony immunoblotting and the Biken assay, however, none of these tests demonstrated the presence of the putative toxin. Southern blot analysis and hybridisation with the GM1 oligonucleotide was also used for probe-negative, but GM1-ELISA-positive strains, but no hybridising fragments were detected.

4.2.3 The cytotoxins of *V. cholerae* non-O1 and *V. mimicus*

The presence of cytotoxin which masks CT production has been described above 4.2.1a. Strains of *V. cholerae* non-O1 and *V. mimicus* produced cytotoxic effects on all three cell lines, Y1, Vero and HeLa, used in the present study. Six patterns of cytotoxin production were observed using these 3 cell lines, indicating that more than one cytotoxin was produced by these strains. The most common pattern (47%) was cytotoxicity on all 3 cell lines. There was no apparent correlation of cytotoxic pattern with serogroup, however, cytotoxin production may be important for virulence as representatives of all the serogroups commonly

isolated from patients with diarrhoea (O2, O5, O13 and O34) were cytotoxic on Y1 cells.

The Vero cell line has been used to detect the Vero cytotoxin (VT) of *E. coli* (Konowalchuk *et al.* 1977; Scotland *et al.* 1980). VT belongs to a group of toxins which are closely related to the Shiga toxin of *Shigella dysenteriae* type 1. VT causes cell death of Vero cell monolayers; however, the cytotoxin produced by *V. cholerae* non-O1 strains in the present study was not neutralised by anti-VT. Therefore, the cytotoxic effect was probably not due to the activity of VT or VT-like toxins. A previous study reported the presence of a specific cytotoxin related to VT or Shiga-like toxin. The toxin appeared to be demonstrated in cell lysates of *V. cholerae* and *V. parahaemolyticus*, using cytotoxicity tests and neutralisation assays with anti Shiga-toxin antiserum (O'Brien *et al.* 1984). VT production could not be detected on Vero cells, in the present study, however, if VT and a second cytotoxin were produced simultaneously by *V. cholerae* non-O1, the VT effect could have been "masked" (similar to the masking of CT). Therefore, strains of *V. cholerae* non-O1 and *V. mimicus* were examined for the presence of VT genes using a combined VT1 and VT2 polynucleotide probe, however, none of the strains hybridised with this probe.

4.2.3a Vacuolating toxin production on Vero cells.

As well as the cytotoxic effect another distinct irreversible heat-labile effect was the vacuolation of Vero cell cytoplasm. The vacuolating toxin was produced on Vero cells by 16% of *V. cholerae* non-O1 strains, this was most commonly associated with a shrivelled appearance on Y1 cells, with no observed effect on HeLa cells. There was no apparent correlation between production of vacuolating toxin and source of isolates or serogroup. Although there have been no other reports of vacuolating toxin production by *V. cholerae* or *V. mimicus*, a similar vacuolation in tissue culture cell assays has been reported for *Helicobacter pylori* (associated with gastritis and duodenal ulcers) (Cover *et al.* 1990; Cover *et al.* 1991; Schmitt and Haas 1994) and the food poisoning organism *Bacillus cereus* (Hughes *et al.* 1988).

It is may be of significance that both these organisms are involved in causing forms of gastrointestinal illness. The vacuolating toxin of *V. cholerae* non-O1 strains should be investigated further; in future studies comparisons could be made with the vacuolating toxins of *H. pylori* and *B. cereus*.

4.2.4 Haemolysin(s)

Haemolysins are thermolabile proteins which lyse cells, including red blood cells. Strains of *V. cholerae* and *V. mimicus* were examined for the ability to produce haemolysin(s) using 1% horse blood agar plates. With the established protocol, 90% of strains examined produced haemolysin(s). Strains produced ß-haemolysin, α-haemolysin and a possible third haemolysin which appeared as a double zone. The production of α- and ß-haemolysins by bacteria has been well recognised; however, the basis for the double halo of haemolysis is poorly understood. Although this would require further investigation, it is possible that the double halo effect was caused by strains producing two distinct haemolysins concurrently. This double zone effect does not appear to have been reported previously, however, *V. alginolyticus* also produce this effect (personal communication Dr. Henrik Chart).

V. cholerae and *V. mimicus* were also tested for haemolysis of calf, sheep, human and rat rbc's, and for cytotoxicity on Y1 cells. The observation that test strains produced haemolysins which were able to lyse a range of red cell types, suggested that the haemolysin(s) were probably non-specific lysins. Haemolysin(s) lysed rat cells in preference to those of guinea pig, human, calf and sheep, the basis for this remains unknown but may relate to the numbers or types of haemolysin receptors present on the various red blood cells.

An attempt was made to purify and raise antibodies to the haemolysin of *V. cholerae* O2. The poor rabbit immune response to the antigen challenge was thought to be as a result of the very low amounts of actual protein toxin used. Had antibodies to *V. cholerae* toxin been available, this would have been a useful tool to investigate the haemolysin(s) and

cytotoxin(s) produced by other *V. cholerae* non-O1 and the closely related *V. mimicus*.

Previous studies have found that strains of *V. cholerae* non-O1 produce a haemolysin which is indistinguishable from haemolysin produced by strains of *V. cholerae* O1 biotype El Tor (Brown and Manning 1985; Yamamoto *et al.* 1986; Ichinose *et al.* 1987). Also, that strains of *V. mimicus* produce a haemolysin which has partial identity with these haemolysins (Sasahara 1993 #1151; Shinoda *et al.* 1993; Uchimura *et al.* 1993). Although the role of haemolysin in pathogenesis is still uncertain, haemolysins are possible virulence factors, particularly for *V. cholerae* non-O1 and *V. mimicus* that do not produce CT.

4.2.4a Iron regulation of haemolysin.

The synthesis of several haemolysins are iron regulated, with maximal expression of haemolysin occurring at very low iron concentrations. During pathogenesis, haemolysins may involve the release of iron-containing compounds such as haem available to pathogenic bacteria. The role of iron in the expression of haemolysins requires the bacteria under investigation to be grown in media with either low levels of available iron, or in media which causes apparent low iron conditions to occur. Whether or not bacteria are growing under conditions of low iron can be assessed by looking for the production of low-iron-inducible chelators such as enterobactin or aerobactin. In the present study, the ability of *V. cholerae* non-O1 to produce haemolysin in the light of expressing the iron chelators, enterochelin and aerobactin, was examined. *V. cholerae* non-O1 did not produce aerobactin, but did produce enterochelin. The medium Tris-succinate was used to give apparent conditions of iron shortage, with the sole carbon source, succinate, causing iron shortage in the cytochrome system. The growth of bacteria in Tris-succinate medium was compared with Tris-succinate medium with added iron in the form of $FeCl_3$. Strains of *V. cholerae* non-O1 were grown in this medium statically and with aeration. In general, strains grown statically produced haemolysin but not enterochelin. This suggested that the bacteria were not growing under conditions of iron

deficiency. However, aerating these cultures and providing a highly aerobic culture caused strains to produce enterochelin but not haemolysin. This suggested that the haemolysin was not induced by conditions of low iron. For other strains, both enterochelin and haemolysin were produced at the same time, it was concluded that production of haemolysin was not iron-regulated.

4.2.5 High affinity iron uptake

Iron is an essential element for most aerobic organisms. Most pathogenic bacteria possess at least one high affinity iron uptake system, which involves the production of low molecular weight, high-affinity iron chelating molecules, known as siderophores. Siderophores have extremely high affinities for iron and are synthesised in response to iron limitation. The two major siderophores are the phenolic compound enterobactin (or enterochelin) and the hydroxamate siderophore aerobactin.

In the present study, strains of *V. mimicus* were found not to produce enterochelin; however, 74% of *V. cholerae* non-O1 strains did produce this siderophore. There was no obvious association of enterochelin production with source of isolate or with serogroup. In contrast, none of the *V. cholerae* strains were positive for aerobactin, but 90% of *V. mimicus* produced aerobactin. The ability to produce aerobactin has been linked to virulence for invasive bacteria such as *E. coli* which have been shown to express an aerobactin-mediated iron uptake system. However, when aerobactin positive strains of *V. mimicus* were tested in BALB/C mice, none showed any adverse reaction. The production of aerobactin by *V. mimicus* did not appear to be linked to virulence in mice. The production of aerobactin appears to be exclusive to *V. mimicus* and the production enterochelin appears to be exclusive to *V. cholerae* strains therefore, these tests are discriminatory between *V. mimicus* and *V. cholerae*.

A recent study has identified the siderophores from *V. hollisae* and *V. mimicus* as aerobactin, this appears to be the first report of aerobactin production outside the family

Enterobacteriaceae except for a halophilic pseudomonad (Okuji and Yamamoto 1994). Previous studies have demonstrated that *V. cholerae* produces a phenolic siderophore related to enterochelin, known as vibriobactin (Payne and Finkelstein 1978; Griffiths *et al.* 1984). In addition haemolysin (Stoebner and Payne 1988) is maximally expressed under iron-limiting conditions. The present study did not find that haemolysin was iron regulated; however, it is possible that more than one haemolysin is expressed by *V. cholerae* and that not all haemolysin(s) are iron-regulated.

4.2.6 The heat stable enterotoxin (NAG-ST)

NAG-ST may be an important virulence factor of CT-negative strains of *V. cholerae* and *V. mimicus*. NAG-ST producing strains of *V.cholerae* non-O1 have been implicated in an epidemic in a Khmer camp in Thailand (Bagchi *et al.* 1993) and human volunteer studies have shown that NAG-ST positive strains can cause diarrhoea of a severity comparable to that of cholera (Morris *et al.* 1990). Heat-stable enterotoxins are recognised virulence factors of *E.coli* and *Yersinia enterocolitica* and NAG-ST belongs this family of enterotoxins. These enterotoxins probably evolved from a common ancestral gene (Arita *et al.* 1986; Takao *et al.* 1985; Ogawa *et al.* 1990) and transpostion may be a possible mechanism for the spread of these ST genes, as the gene for *E.coli* STI has been located within a transposon, Tn1681 (So and McCarthy 1980). In addition it has suggested that the NAG-ST may have originated in *V. mimicus* (Pal *et al.* 1992; Ramamurthy *et al.* 1993).

In the present study the production of NAG-ST by strains of *V. cholerae* non-O1 could not be demonstrated in the infant mouse assay. *V. cholerae* non-O1 gave indeterminate FA ratios and there was no obvious fluid accumulation seen, even with a control NAG-ST positive *V. cholerae* strain (NAG-82E). The infant mouse assay was traditionally used to detect STA of *E. coli* (Dean *et al.* 1972) and has been successfully used to detect NAG-ST producing *V. cholerae* non-O1 in a previous study (Arita *et al.* 1986; Spira *et al.* 1979).

The presence of VM-ST, which appears to be identical to NAG-ST, in *V. mimicus* has also been demonstrated (Honda *et al.* 1985). The failure to detect NAG-ST in the present study may be due to the growth conditions used, however, several different cultural conditions including those suggested by Arita *et al.* were tried without success.

Although NAG-ST production could not be demonstrated strains of *V. cholerae* non-O1 could be screened for the presence of the NAG-ST gene. Initially a commercially available SNAP (synthetic nucleic acid probe) developed for the detection of *E. coli* ST was used, however, this probe did not hybridise with any of the strains of *V. cholerae* examined. Although the amino acid sequences of NAG-ST and VM-ST toxin were similar to that of *E. coli* ST (Takao *et al.* 1985) the nucleotide sequences of the genes encoding them are distinctly different, with only 50% homology (Ogawa *et al.* 1990). This probably explains the failure to detect NAG-ST using the SNAP-ST probe, other studies using *E. coli* ST probes also failed to detect NAG-ST (Sommerfelt *et al.* 1988; Seriwatana *et al.* 1987).

Two oligonucleotides were synthesised based on sequences published by Hoge *et al.*, a mixed oligonucleotide probe deduced from the amino acid sequences of *E. coli* STh and STp (Hoge *et al.* 1990) and Ogawa *et al.*, a nucleotide sequence based on the NAG-ST gene (Ogawa *et al.* 1990). The mixed oligonucleotide probe of Hoge *et al.* has been used to study *V. cholerae* non-O1 isolates from Thailand (6.8% were NAG-ST positive) and from Mexico and USA (none were positive for NAG-ST) (Hoge *et al.* 1990). However, the hybridisation experiments using the probe described by Hoge *et al.* in the present study were not satisfactory because the *V. cholerae* NAG-82E control strain did not hybridise. In contrast, it was found that the probe described by Ogawa *et al.* gave a clear positive result with the *V. cholerae* NAG-82E control strain.

Using the Ogawa oligonucleotide, all *V. cholerae* O1 were NAG-ST probe-negative, as were all CT-positive *V. cholerae* non-O1. These findings, that strains possessing the gene for NAG-ST do not possess the gene for CT and vice versa, are in agreement with Pal

et al. (Pal *et al.* 1992). However, one study has identified the NAG-ST toxin in 1 of 197 isolates of *V. cholerae* O1 (Takeda *et al.* 1991).

In the present study, 3% of the CT-negative *V. cholerae* non-O1 strains hybridised with the Ogawa NAG-ST oligonucleotide. Of the environmental isolates of *V. cholerae* non-O1, 5% were NAG-ST probe-positive, compared with only 1% NAG-ST probe-positive from clinical sources. There also appeared to be a correlation with serogroup O14; all 7 *V. cholerae* strains belonging to the O14 serogroup (4 strains from the environment and 3 strains of human origin) were NAG-ST probe-positive. In contrast to the O14 serogroup, NAG-ST probe-positive strains were the exception in other serogroups. Recently Pal *et al.* used a 271 bp cloned NAG-ST DNA probe to assess the prevalence of NAG-ST among environmental strains isolated in Calcutta (Pal *et al.* 1992). They found 2.3% (12/521) of strains hybridised and suggested that there might be a correlation between serogroup and NAG-ST. The present study suggests the correlation of the NAG-ST virulence factor with *V. cholerae* serogroup O14.

As well as a correlation with the O14 serogroup of *V. cholerae* non-O1, NAG-ST (or VM-ST) was associated with the species *V. mimicus*. Nearly 12% of *V. mimicus* strains hybridised with the NAG-ST probe, interestingly none of 3 *V. mimicus* strains which belonged to serogroup O14 hybridised with the NAG-ST probe. A study using thirteen strains of *V. mimicus* (Ramamurthy *et al.* 1994), demonstrated that these organisms were capable of producing several toxins simultaneously; including CT, NAG-ST, TDH and cytotoxin. They suggested that *V. mimicus* may act as a "courier" of virulence factors among vibrios using transposons as a means of transferring genetic material. In particular it was suggested that *V. mimicus* acts as an important reservoir for NAG-ST genes. Although, the present study found that the NAG-ST gene was present more frequently in *V. mimicus* (12%) than in *V. cholerae* non-O1 (3%), there is insufficient evidence to suggest that *V. mimicus* is the main reservoir for NAG-ST. Both *V. mimicus* and *V. cholerae* non-O1 from both clinical and environmental

sources had the capacity to express several toxins and these toxins could be expressed concurrently. This may suggest that both species could act as reservoirs of toxins, or that these factors are required for these organisms to exist successfully in their natural environment.

4.2.6a Southern blot analysis of the NAG-ST gene.

The NAG-ST gene appears to be a stable factor which is chromosomally encoded. Genomic DNA preparations were made and digested with *Hin*dIII. The Southern blots were hybridised with the NAG-ST oligonucleotide probe of Ogawa *et al.* and found to hybridise with a single chromosomal restriction fragment, of between 2 and 19 kb. All *V. cholerae* non-O1 strains gave single bands of different sizes, with the exception of an unidentifiable (O?) *V. cholerae* strain (E47276) that had 2 hybridising fragments (8 kb and 2.3 kb), indicating that this strains possessed 2 copies of the NAG-ST gene. The NAG-ST probe-positive strains of *V. cholerae* were differentiated into 8 groups by the size of the hybridising fragment. All strains of the O14 serogroup, regardless of source or geographical origin gave a single hybridising fragment of 19 kb. The presence of the same size of hybridizing fragment in all O14 strains, and the fact that this fragment does not occur in other serogroups, may indicate that NAG-ST positive O14 represents a single clone. This could explain the close association of this serogroup of *V. cholerae* with NAG-ST. Two patterns (H5 and H6) were found in strains from a variety of serogroups. The incorporation of the NAG-ST gene at identical chromosomal sites, possibly arising by transposition, may account for the serogroup heterogeneity found within these patterns.

It would also be interesting to analyse the NAG-ST positive strains of *V. mimicus* found in the present study by Southern blot analysis. A comparison of the size of hybridising fragment may begin to clarify the relationship, if any, between the NAG-ST positive *V. cholerae* non-O1 and *V. mimicus*.

4.2.7 Colonisation factors

The ability of bacteria to adhere to host tissues is a recognised pathogenic mechanism, and the use of cultured cell lines, such as HEp-2, have been useful for studying bacterial adhesion *in vitro*. Recently, it has been suggested that pathogenic *V. cholerae* non-O1 could be distinguished from non-pathogenic strains by adhesion on the Caco2 cell line (Panigrahi *et al.* 1990). In the present study, both Caco2 and HEp-2 tissue culture cell lines were used to test *V. cholerae* non-O1. The adhesion tests on both, HEp-2 and Caco2 cell lines, were best performed as 3 h rather than the conventional 6 h test used for *E. coli* as the *V. cholerae* strains produced cytotoxins which destroyed the cell monolayer. The strains were also tested for haemagglutination using calf, guinea pig, human and rat rbc's. The different growth conditions did not make any obvious difference in the adhesion results obtained on the cell lines, however, the presence of mannose did affect the haemagglutination of rbc's. Strains tested gave mannose-sensitive (MS) haemagglutination on guinea pig rbc's and mannose-resistant (MR) haemagglutination on human rbcs. The adhesion tests were repeated in the presence of sugars other than mannose; no inhibition of adhesion due to mannose, fucose or rhamnose was found. The present study found no correlation between haemagglutination and adhesion. Neither the possession of smooth LPS, nor flagella appeared to be necessary for adhesion. Both environmental and diarrhoea-associated strains were found to adhere avidly, not only to both cell lines, but also to glass only, indicating that the adherence is non-specific. The use of the Caco2 cell, a moderately differentiated human colonic adenocarcinoma, which has proved a useful *in vitro* model for adhesion of pathogens such as *Listeria monocytogenes* and *E. coli*, did not appear to distinguish pathogenic *V. cholerae* non-O1 from non-pathogenic strains as suggested by Panigrahi *et al.* (Panigrahi *et al.* 1990).

Colonisation of the epithelial mucosa of the small intestine appears to be a critical step in the ability of organisms, such as *V. cholerae* O1 and *V. cholerae* non-O1, to elicit diarrhoea (Spira and Fedorka-Cray 1983). A number of factors have been associated with

the colonisation of epithelial cells, these include; motility through a single polar flagellum, haemagglutinins, outer membrane proteins, lipopolysaccharides and pili.

Early studies suggested that non-motile variants of *V. cholerae* O1 were less virulent and that the flagellum plays a key role in adhesion by aiding both, penetration of the mucous barrier and colonisation of the epithelial surface (Freter 1981; Attridge and Rowley 1983). Other studies have failed to corroborate the findings that non-motile strains were less virulent (Teppema *et al.* 1987). Attridge and Rowley demonstrated both binding and motility functions associated with the flagellum (Attridge and Rowley 1983). The present study suggests that motility is not a requirement for adhesion, however, strains which possess flagella can be non-motile hence the flagellum may still play a role in adhesion.

Previous studies have examined the distribution of a variety of haemagglutinins as potential colonisation factors in strains of *V. cholerae* O1 and non-O1 (Booth and Finkelstein, 1986). It has been suggested that cell-associated mannose-sensitive, fucose-sensitive, and mannose-fucose-resistant haemagglutinins may be virulence factors in pathogenic *V. cholerae* (Hanne and Finkelstein 1982; Jones and Freter 1976; Bhattacharjee and Srivastava 1978; Attridge and Rowley 1983). However, Booth and Finkelstein concluded that although cell-associated haemagglutinins were widely distributed in *V. cholerae* O1 and non-O1, these factors are not sufficient for virulence (Booth and Finkelstein 1986). In the present study there did not appear to be an association with haemagglutination and adhesion on the HEp-2 and Caco2 cell lines.

The most recent research suggests that pili are the critical factor in colonisation. In the present study no pili were seen by transmission electron microscopy, however, CT-positive strains of *V. cholerae* non-O1 were shown by PCR to possess the gene for the toxin co-regulated pili. Several groups have reported the presence of pili in *V. cholerae* O1 (Ehara *et al.* 1987; Hall *et al.* 1988; Taylor *et al.* 1987; Pearson *et al.* 1993), *V. cholerae* non-O1 (Honda *et al.* 1988) and *V. mimicus* (Uchimura and Yamamoto 1992). The best characterised pilus of

V. cholerae is the toxin co-regulated pilus (TCP) (Taylor *et al.* 1987; Attridge *et al.* 1993). These hydrophobic pili have been observed to form large bundles under the electron microscope and the expression of these TCP bundles causes autoagglutination, sedimentation in broth culture and the ability to haemagglutinate mice rbc's in the presence of fucose or mannose. The role of TCP in colonisation has been established for both *V. cholerae* O1 biotypes in volunteer studies; mutant strains which did not produce pili failed to colonise the intestine and failed to produce an effective immune response (Herrington *et al.* 1988). In the present study, CT-probe-positive strains of *V. cholerae* non-O1 were shown, by PCR, to possess the gene for *tcpA*, however the CT-negative strains of *V. cholerae* non-O1 and *V. mimicus* did not appear to possess the *tcpA* gene, however, as these strains adhere to the tissue culture cells they presumably do so by some other mechanism.

The present study demonstrated that all *V. cholerae* O139 possess a *tcpA* identical to El Tor *tcpA* and that a similar association exists between other non-O1 serogroups and type of *tcpA*. However, confirmation of this would require greater numbers of CT positive strains belonging to these serogroups and originating from different geographical areas to be tested. Based on restriction fragment patterns by pulse field electrophoresis and on PCR directed against *ctxA* and *tcpA*, it has been suggested that strains of serogroup O139 are El Tor variants (Hall *et al.* 1993). In the present study, however, *ctxB* and *tcpA* gene amplicons of O139, O1 biotype El Tor and certain O6 and O? strains are indistinguishable in size. Rather than suggesting that CT positive non-O1 are variants of O1, it may be that transfer of CT and TcpA genes has occurred several times from O1 to non-O1 strains and that CT positive strains of a particular serogroup may be members of a clone resulting from this event.

The mechanisms of colonisation of *V. cholerae* non-O1 and *V. mimicus* are not fully characterised, it may be that, similar to diarrhoeagenic *E. coli*, more than one mechanism of adherence will be found.

4.2.8 Lipopolysaccharide (LPS) and Outer membrane proteins (OMP)

LPS and OMP have been implicated as virulence factors in *V. cholerae* diarrhoea; both have been associated with colonisation of the epithelial mucosa of the small intestine (Spira and Fedorka-Cray 1983). The LPS and many of the OMP of *V. cholerae* are highly immunogenic (Manning and Haynes 1984; Kabir 1986; Sears *et al.* 1984). The outer membranes of vibrios contain several proteins (OMPs) which are strongly antigenic, *V. cholerae* O1 possess 4 different OMP in the 25 - 45 kda range. Outer membrane proteins were examined for strains of *V. cholerae* non-O1.

In the present study *V. cholerae* non-O1 and *V. mimicus* showed considerable diversity in their OMP, with the majority having a major OMP at around 38 kda. Strains of *V. cholerae* belonging to O2 and O27 appeared not to express porins. This is difficult to explain, but has been observed in *V.cholerae* O1 previously (Bertram 1990). The OMP of *V. cholerae* non-O1 and *V. mimicus* may reflect the types of toxin elaborated by particular strains, for example, strains which produce particular toxins may have the same OMP profiles because of the need to excrete proteins or toxin. The lack of a porin in the *V. cholerae* O2, particularly if this was shown to be serogroup related, may also be of interest as the O2 serogroup does appear to be associated with diarrhoeal disease. Further investigation would be required into both these aspects.

Previous studies have found a major protein which migrated on SDS-PAGE gels as a band of 48 kda, which was common to all strains of *V. cholerae* O1 (Kabir 1986). Kelley and Parker found a 45 kda major protein which appeared to be the major structural protein of the outer membrane (Kelley and Parker 1981). The OMP of *V. cholerae* were compared to the OMP of other gram-negative bacteria and were found to have a distinct OMP pattern (Manning *et al.* 1982). Strains of *V. cholerae* O1 generally contain OMP in the range 25 - 45 kda, and also additional minor proteins and *V. cholerae* non-O1 appear to be more heterogeneous; a protein at 25 kda appears to be common to all strains of *Vibrio* (Manning and

Hayes 1984). The present study also found a single major OMP (although this was at approximately 38 kda), and also some unusual OMP profiles with no apparent major OMP, this is extremely unusual and should be investigated further.

The LPS of *V. cholerae* O1 and O139 are important protective antigens and, as these serogroups are the only *V. cholerae* known to cause epidemic cholera, are considered virulence factors. The present study indicates the association of certain non-O1 and non-O139 serogroups, such as O2, and O5, with diarrhoeal disease and also the association of certain serogroups with the possession of toxin genes; O1 and O139 with CT, and O14 with NAG-ST.

Although the role of LPS as an adhesive factor has not been established, antibodies produced against *V. cholerae* LPS are protective in animal models, and the observed protection may involve interference with LPS during mucosal colonisation (Booth *et al.* 1986). However, the present study found that the possession of smooth LPS was not required for adhesion on HEp-2 and Caco2 tissue culture cell lines, indicating that long chain LPS is not a prerequisite for adhesion.

In the present study, SDS-PAGE analysis of the LPS of *V. cholerae* non-O1 has proved useful in confirming outbreaks of otherwise unidentifiable strains and for clarifying problems with identification of serogroups, such as O26. The appearance of *V. cholerae* LPS is unusual, if a "ladder" structure can be seen it is usually irregular, however, the pattern observed within a particular serogroup is indistinguishable. Several serogroups, though did show marked heterogeneity, rather than identity of LPS. On further investigation a number of new serogroups were identified and antisera raised to these presumptive serogroups. The ability of strains of *V. cholerae* to express long-chain LPS is only poorly understood, although strains belonging to serogroups O1 and O139 are thought to express LPS, these serogroups proved to be among the serogroups from which LPS could not be readily identified. The "smear" seen on SDS-PAGE gels of these serogroups may be due to the presence of capsular material.

CONCLUSIONS

Vibrio cholerae non-O1 and the closely related *V. mimicus*, were characterised by serogroup and biotype and examined for factors related to virulence. Serogrouping was a reliable means of differentiating strains, although only 30% of strains could be identified using sera raised against O2 - O83 and O139, this would doubtless be improved by the expanded scheme of Shimada *et al.* which recognises 141 serogroups (O1 - O140). All *V. cholerae* O1, all sub-serogroups (Ogawa, Inaba or Hikojima) and both biotypes (classical and El Tor) were typable within the scheme of Sakazaki and Donovan. The serogrouping scheme also applies to the closely related *V. mimicus* but not to any of the halophilic vibrios.

Strains were examined for the possession of genes and the production of toxins as well as cell-associated factors. Cholera toxin (CT), the main virulence factor responsible for the dramatic symptoms of epidemic cholera, is produced by *V. cholerae* O1 and O139, but is rarely found in *V. mimicus* and *V. cholerae* belonging to other serogroups. The CT-positive strains were differentiated by Southern blot analysis of CT; most strains had a single hybridising fragment suggesting the presence of a single copy of the CT gene. The CT-positive strains belonging to serogroups O23 and O139 strains had more than one hybridising fragment indicating the presence of more than one CT gene. *V. cholerae* O139 also showed variation in the size of hybridising fragment, suggesting that O139 strains were not clonal in origin. The CT genes of the only CT-positive *V. mimicus* strain appeared to be distinct from the CT genes of *V. cholerae* non-O1 both in the size of the single hybridising fragment in Southern blot analysis and in the fact that it could not be detected by PCR for CT.

The heat-stable enterotoxin (NAG-ST) is another established virulence factor which has been described. In this study 3% of *V. cholerae* non-O1 possessed the gene for NAG-ST and all *V. cholerae* strains belonging to the O14 serogroup were NAG-ST positive.

By Southern blot analysis of the NAG-ST gene a single identical hybridising fragment was found in the O14 strains. This fragment did not occur in other NAG-ST positive strains, therefore, *V. cholerae* O14 probably represents a single clone. NAG-ST may be important in the virulence of *V. mimicus* as 12% of strains possessed the gene.

The majority of *V. cholerae* non-O1 and *V. mimicus* strains produced cytotoxins, however, none of the strains produced verocytotoxin (VT) or possessed the gene for VT. The low incidence of established toxins (CT, NAG-ST and VT) in *V. cholerae* non-O1, non-O139 and *V. mimicus* suggests that other virulence factors are involved. Both *V. cholerae* non-O1 and *V. mimicus* were capable of producing many extracellular products, including cytotoxins and haemolysins. These factors were often produced concurrently and led to problems in creating the optimal conditions and choosing the appropriate assay for the factor being investigated. Over 80% of strains produced cytotoxin(s) as detected by Y1 cells, and nearly 50% of strains were cytotoxic on all three cell lines (Y1, HeLa and Vero). The cytotoxic effect masked the cytotonic effect of CT produced by *V. cholerae* non-O1, non-O139. Haemolysin(s), α-, ß- and an unusual double zone haemolysin, were produced by 90% of *V. cholerae* non-O1 and *V. mimicus*. The role of cytotoxins and haemolysins in diarrhoeal disease needs to be further elucidated.

Two novel toxins were found in this study. The first was a cytotoxin which produced vacuolation of Vero cell cytoplasm and was expressed by 16% of strains; this toxin may be related to toxins observed in *Helicobacter pylori* and *Bacillus cereus*, which produce a similar vacuolation. The second putative toxin, which was expressed by 19% of strains, was demonstrated in an ELISA assay, this "toxin" was distinct from the cytotoxin and vacuolating toxin, had the ability to bind ganglioside GM1 and shared antibody binding sites with CT.

High affinity iron chelating compounds (siderophores) can be important in bacterial virulence; both *V. cholerae* non-O1 and *V. mimicus* express siderophores. Seventy-four percent of *V. cholerae* non-O1 produce enterochelin while 90% of *V. mimicus* produce

aerobactin. The production of aerobactin is unusual in *Vibrio spp.* and has been linked to the virulence of invasive bacteria.

Adhesion by more than 90% of *V. cholerae* non-O1 and *V. mimicus* strains was demonstrated on both HEp-2 and Caco2 cell lines. However, the mechanism by which this adherence occurs remains to be elucidated. The present study found that neither smooth LPS, nor flagella are essential for adhesion. The toxin coregulated pili probably have a role in colonisation for CT-positive strains; CT-positive strains of *V. cholerae* non-O1 were shown by PCR to possess the gene for the toxin coregulated pilus (*tcpA*). Of the two types of *tcpA* (identical to either *tcpA* found in classical or El Tor biotypes of *V. cholerae* O1), all *V. cholerae* O139 possessed a *tcpA* identical to El Tor-type of *tcpA*. A similar association also appears to exist between other CT-positive non-O1 serogroups and type of *tcpA*. However, CT-negative strains did not possess the *tcpA* gene and pili were not seen by transmission electron microscopy, therefore some other mechanism must be involved in the adhesion of these strains.

V. cholerae non-O1 and *V. mimicus* also showed considerable diversity in their outer membrane proteins (OMP), with the majority having a major OMP at around 38 kda. Strains of *V. cholerae* belonging to O2 and O27 were particularly unusual because they appeared not to express porins.

The somatic antigen may be a useful indicator of diarrhoeagenic potential within *V.cholerae* non-O1. Serogroups, O2, O5, O9, O11, O13, O24, O34, O37 and O139, were associated with clinical isolates and serogroup O19 was commonly isolated from the environment. The most common serogroups of *V. cholerae* associated with diarrhoeal disease, excluding O1 and O139, were serogroups O2 and O9. The LPS of *V. cholerae* O1 and O139 are important protective antigens and are considered to be virulence factors. The present study indicates the association of certain serogroups with the possession of toxin genes (O1 and O139 with CT and O14 with NAG-ST) and also the association of certain non-O1, non-O139 serogroups, with diarrhoeal disease.

Many of the factors described in this study need further investigation, in particular the two novel toxins the vacuolating toxin and the ELISA "toxin". The vacuolating toxin could be compared to the toxins of *H. pylori* and *B. cereus* which cause similar vacuolation in tissue culture cells. Further work could also be done on differentiating the haemolysins and cytotoxins produced by these strains, the "double zone" haemolysin(s) is unusual and may be of particular interest. The mechanisms of colonisation and further investigations into the LPS and OMP of *V. cholerae* non-O1 and *V. mimicus* and possible iron regulation of these factors could be undertaken. Future studies could focus on the virulence attributes of specific serogroups found to be associated with diarrhoea, such as O2, O5, O9, O24 and O37.

The pathogenicity of *V. cholerae* and *V. mimicus* appears to be multifactorial and further work could be done to identify these factors and establish further correlations with serogroup. None of the virulence factors produced by these strains, including CT, is found exclusively in human rather than environmental isolates. The presence of toxins and cell associated factors in environmental strains could account for human infections as water and seafood appear to be the main vehicles of transmission for vibrio-associated infections. It will probably not be possible to identify a single characteristic that differentiates virulent strains of *V. mimicus* and *V. cholerae* non-O1 from avirulent strains. It is more likely that heterogeneous patterns of virulence, similar to the heterogeneity seen among diarrhoeagenic *E. coli*, will be found.

Appendix 1

A. Media

All media were prepared by the Media Department, CPHL. Tests were carried out at 37°C unless, otherwise stated.

References:

Cowan and Steel's Manual for the identification of Medical Bacteria. Third Edition. Edited by G.I. Barrow & R.K.A. Feltham. Cambridge University Press.

Edwards and Ewings Identification of Enterobacteriaceae. Fourth Edition. W.H. Ewing. Elsevier.

A.1 Culture maintenance media

Nutrient agar medium

25 g Oxoid Nutrient Broth No2 CM67

15 g Japanese agar

1 l distilled water.

Dorset Egg medium

25 g sterilised egg mixture (Lab-Lemco powder)

12.5 % NaCl

1 l distilled water.

Freeze-drying medium

Difco nutrient broth containing 0.5% inositol

A.2 Biochemistry

Alkaline Peptone Water

10 g Oxoid peptone L37

5 g NaCl

1 l distilled water, pH 9.2

Peptone Water Sugars with Andrade's indicator

900 mls peptone water

10 mls Andrade's indicator, pH 7.5.

The sugars were dissolved and sterilised by filtration, then added aseptically to the sterilised peptone water with indicator. The following sugars were used; adonitol, arabinose, cellobiose, dulcitol, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose and xylose. The glucose PW also contained an inverted inner (Durham) tube to test for gas. Sugars used for testing vibrios also had 1% NaCl added. Positive = pink (gas bubble in Durham tube). Negative = colourless, after 14 days.

ONPG (o-nitrophenyl-ß-D-galactopyranoside)

250 mls ONPG solution (6 g ONPG, 1 l 0.01 M Na_2HPO_4, pH 7.5)

750 mls peptone water.

Positive = yellow colour in solution. Negative = solution remains colourless, after 2 days.

Citrate utilization

Christensen's citrate

3 g Sodium citrate

0.2 g Glucose

0.5 g Yeast Extract

0.1 g L-cysteine hydrochloride

1 g KH_2PO_4

5 g NaCl

20 g Agar

6 mls Phenol red (0.2% solution)

1 l distilled water, pH 6.8 - 6.9.

Positive reaction = pink colouration of slope. Negative = original yellow colour of slope, after 7 days.

Simmons' citrate

5 g NaCl

0.2 g $MgSO_4.7H_2O$

1 g $NH_4H_2PO_4$

1 g K_2HPO_4

2 g Citric acid,

20 mls bromothymol blue (0.4% solution)

20 g Agar

1 l distilled water.

Positive = bright blue colouration of slope. Negative = original blue/green colour of slope, after 7 days.

Acetate utilization

Sodium acetate

This medium is the same as Simmons' citrate agar, except that 0.25% sodium acetate is used instead of citrate. Postive = bright blue colour. Negative = original blue/gree colour of slope, after 7 days.

Urease activity

Urea medium

1 g Peptone

5 g NaCl

2 g KH_2PO_4

20 g Agar

1 l distilled water, pH 6.8.

Sterilise then add 1 g glucose and 6 mls phenol red (0.2% solution), steam for 1 h before adding 100 mls sterile urea (20% solution). Positive = pink colour. Negative = original colour of slope, after 7 days.

Arginine dihydrolase production, lysine and ornithine decarboxylase production

Moller's medium

5 g Peptone

5 g meat extract

5 mg pyridoxal

0.5 g glucose

2.5 mls bromothymol purple (0.2% solution)

2.5 mls Cresol red (0.2% solution) in 1 l distilled water.

For use add 1% L-arginine hydrochloride or 1% L-lysine hydrochloride or 1% L-ornithine hydrochloride as required, adjust to pH 6. A control tube, with no addition, is included in the test.

Positive = purple colour. Negative = yellow colour (NB: the control tube must be yellow), after 4 days.

Indole production.

The strain to be tested is grown in peptone water for 2 days, then a few drops of *Kovac's reagent* (5 g *p*-dimethylaminobenzaldehyde dissolved in 75 mls amyl alcohol, 25 mls conc HCl) is added. Positive = dark pink ring. Negative = yellow ring.

Hydrogen sulphide (H2S) production in glucose iron agar

3 g beef extract

3 g yeast extract

15 g peptone

5 g proteose peptone

10 g glucose

0.2 g ferrous sulphate

1 l distilled water.

Positive = blackening. Negative = original red/orange colour of slope, after 7 days.

Growth in potassium cyanide (KCN).

KCN base

3 g peptone

5 g NaCl

0.225 g KH_2PO_4

5.64 g $Na_2HPO_4.2H_2O$

1 l distilled water, pH 7.6.

For use add 1.5 mls 0.5% KCN in sterile distilled water to 100 mls of base. The strain to be tested is inoculated into 1 ml base containing KCN and 1 ml base without KCN in seperate bijou bottles. After 2 days the bottles are examined for growth. Positive = growth in the base with and without KCN. Negative = growth in base without KCN only.

Combined Malonate utilization and PPA test (deamination of phenylalanine to phenylpyruvic acid)

2 g $(NH_4)_2SO_4$

0.6 g K_2HPO_4

0.4 g KH_2PO_4

2 g NaCl

3 g sodium malonate

2 g DL-phenylalanine

1 g yeastrel

5 mls bromothymol blue (0.5% w/v)

1 l distilled water.

The medium is inoculated and incubated overnight.

Malonate test

Positive = blue. Negative = original green colour of solution.

PPA test

After recording malonate result, acidify with 10 N HCl (few drops) until the colour of the medium changes to yellow. Add 4 - 5 drops of 10% (w/v) ferric chloride. Positive = dark green. Negative = yellow (may be cloudy).

Gluconate oxidation

1.5 g peptone

1 g yeastrel

1 g K_2HPO_4

40 g potassium gluconate in 1 l distilled water, pH 7.

1 ml volumes in tubes are inoculated with the strain(s) to be tested and incubated for 2 days. A "Clinitest" (Miles Ltd, Ames Div, Slough) tablet for reducing sugars is added to each tube. Positive = dark green or purple or brown precipitate. Negative = bright blue.

Aesculin hydrolysis

Aesculin slopes

40 g ox bile dehydrated

1 g aesculin

0.5 g ferric citrate

15 g agar

1 l nutrient broth

Positive = blackening of the slope. Negative = original grey colour of slope, after 14 days.

Gelatin hydrolysis.

3 g beef extract

5 g peptone

120 g gelatin

1 l distilled water.

Stab inoculate the agar and incubate overnight, leave at adjust to room temperature before examining for liquefaction. Positive = slope is liquefied. Negative = slope remains solid after 28 days at room temperature.

Motility - Craigie tubes. Thiotone agar medium

10 g thiotone,

3 g beef extract

80 g gelatin

5 g NaCl

4 g Shred agar in 1 l distilled water.

The medium is distributed in test tubes containing inner tubes which are open at both ends. The test organisms are stab inoculated into the agar within the inner tube, motile organisms grow down through the inner tube and up through the agar outside the inner tube. Positive = ring of growth outside the inner tube. Negative = no growth outside inner tube, after 14 days.

Hugh & Leifson (H & L). Oxidation-fermentation (OF) test.

2 g peptone

5 g NaCl

0.3 g K_2HPO_4

3 g agar

15 mls bromothymol blue (0.2% solution)

1 l distilled water, sterilise. Then add 1% sterile glucose.

For each test 2 slopes are used. The strain to be tested is stab inoculated into the agar, then one slope is covered with soft yellow paraffin. If the strain is OF positive both slopes turn from green to yellow. Negative = green, after 7 days.

Nitrate reduction

1 g KNO_3 (Analar - nitrite free) in 1 l nutrient broth. Inoculate nitrate broth and incubate overnight. Add 0.5 ml solution A and 0.5 ml solution B. Positive = dark pink to red colour. Negative = no change (NB: this may be because not only has the nitrate been reduced, but the nitrite has been further reduced. In this case add a tiny amount of zinc dust; positive = no change, negative = pink).

Solution A

1.5 mls Dimethyl-α-naphthylamine

178.5 mls distilled water

71.5 mls Glacial acetic acid.

Solution B

2 g sulphanic acid dissolved in 178.5 mls distilled water

71.5 mls Glacial acetic acid.

Vogues-Proskauer (VP) test (acetylmethylcarbonil production).

VP medium

0.1% yeast extract

0.7% trypticase peptone

0.5% phytone peptone

1% glucose

1% NaCl

0.3% agar

1 l of distilled water, pH 7.

Stab inoculate VP agar and incubate overnight at 30°C. Add 0.2 ml solution A and 0.1 ml solution B, read after 15 min at room temperature. Positive = deep red ring. Negative = colourless to yellow.

Solution A

5% α-naphthol in alcohol.

Solution B

40% potassium hydroxide, 0.3% creatine in 100 mls distilled water.

Oxidase activity

1% NNN'N' Tetramethyl-*p*-pheylenediamine dihydrochloride in distilled water. Place drops of reagent on filter paper and using a plastic loop smear a little growth onto the impregnated paper. Positive = deep purple within 30 secs. Negative = no colour change.

A.3 Growth media

Vibrio Phage Typing Agar.

30 g Todd Hewitt Broth (Oxoid)

2 g Yeast extract (Oxoid)

0.01 g Tryptophan

7 g agar No.1 (Oxoid)

1 l distilled water, pH 7.6.

Syncase Sucrose broth (SSB) and *Syncase Glucose broth (SGB)*

5 g Na_2HPO_4

5 g K_2HPO_4

1.18 g NH_4Cl

0.089 g Na_2SO_4

0.042 g $MgCl_2.6H_20$

0.004 g $MnCl_2.4H_20$

0.005 g $FeCl_3.6H_2O$

10 g Casamino acids (Difco)

1 l distilled water.

For Syncase-glucose 0.5%, add glucose 5 g per litre.

For Syncase-sucrose 0.5%, add sucrose 5 g per litre.

Trypticase Soy Broth (TSB)

17 g trypticase peptone

3 g phytone peptone

5 g NaCl

2.5 g K_2HPO_4

2.5 g glucose

1 l distilled water, pH 7.3.

Casamino Yeast Extract broth (CYE)

20 g Casamino acids (Difco)

6 g Yeast Extract (Difco)

2.5 g NaCl

8.7 g K_2HPO_4

1 ml trace salts solution

1 l distilled water, adjust to pH 7.5 with NaOH.

Trace salts solution is 5 g $MgSO_4$, 0.5 g $MnCl_2$ and 0.5 g FeCl3 dissolved in 100 ml H_2SO_4 (0.001 N).

Brain Heart Infusion broth (BHI)

37 g BHI (Oxoid) in 1 l distilled water.

Biken (Modified Elek) Test

Biken Agar

2 g Casamino acids (Difco)

1 g Yeast extract (Difco)

0.25 g NaCl

1.5 g K_2HPO_4

0.5 g glucose

0.05 ml Trace salts solution

1.5 g Noble agar

100 mls distilled water, pH 7.5.

Trace salts solution = 5% (w/v) $MgSO_4$, 2% (w/v) $CoCl_2.6H_2O$ and 0.5% (w/v) $FeCl_3$. Add 0.1 ml lincomycin (13.5 mg/ml) per 15 mls Biken agar.

Nutrient broth

20 g Difco Nutrient Broth

8.5 g NaCl

1 l distilled water.

CFA agar

2% (w/v) agar

1% Casamino acids

0.15% yeast extract

0.005% $MgSO_4$

0.0005% $MnCl_2$.

Tris-succinate medium

5.8 g NaCl

3.7 g KCl

1.1 g NH_4Cl

0.15 g $CaCl_2.2H_2O$

0.10 g $MgCl_2.6H_2O$

0.142 g Na_2SO_4

0.272 g KH_2PO_4

12.1 g Tris base

10 g sodium succinate

800 mls distilled water, adjust pH to 6.8 using conc HCl and make up to 1 litre. Filter sterilise.

A.4 Buffer solutions and miscellaneous reagents

Phosphate buffered saline (PBS)

8 g NaCl

0.2 g KCl

1.15 g Na_2HPO_4

0.2 g KH_2PO_4

1 l distilled water, pH 7.3

Saline

0.9% w/v NaCl in distilled water.

Tris EDTA buffer (TE)

10 mM Tris, 1 mM EDTA, pH 8 and filter sterilise.

Ferric perchlorate solution (for detection aerobactin)

1.52 mls perchloric acid

0.177 g ferric perchlorate

100 mls with distilled water.

Reagents for detection of enterochelin

1) 0.5 M HCl.

2) Nitrite/molybdate reagent (10 g sodium nitrite + 10 g sodium molybdate in 100 mls distilled water).

3) 1 M NaOH.

B. Tissue culture

All reagents for tissue culture were obtained from Flow Laboratories Ltd., Rickmansworth, England.

B.1 Tissue culture cell lines

Y1 adrenal cells Y1, mouse adrenal cortex tumour, ATCC No. CCL79.

Culture medium: Ham's F10.

To 100 mls Ham's F10 add; 12.5 mls horse serum

2 mls foetal bovine serum

2 mls penicillin/streptomycin (5000 units/ml)

0.5 ml glutamine (200 mM)

Amphotericin B (Fungizone) (250 μg/ml).

Vero cells, African green monkey kidney, ATCC No. CCL81.

Culture medium: Medium 199 with Earle's salts.

To 100 mls 199 medium add; 10 mls foetal bovine serum

2 mls penicillin-streptomycin (5000 units/ml)

0.5 ml glutamine

Amphotericin B (Fungizone) (250 μg/ml)

HeLa cells Hela S3 cells, clone of HeLa cervical carcinoma, ATCC No. CCL2.2.

Culture medium: Minimal Essential Medium (MEM) with Eagle's salts.

To 100 mls MEM add; 10 mls foetal bovine serum

2 mls penicillin/streptomycin (5000 units/ml)

1 ml non-essential amino acids (100x)

0.5 ml glutamine (200mM)

Amphotericin B (Fungizone) (250 μg/ml).

HEp-2 cells, carcinoma of larynx, ATCC No. CCL23.

Culture medium: Basal medium eagle (BME) with Hanks salts.

To 100 mls BME add; 15 mls foetal bovine serum

2 mls penicillin-streptomycin (5000 units/ml)

0.5 ml glutamine (200 mM)

Amphotericin B (Fungizone) (250 μg/ml).

Caco2 cells, a colonic carcinoma cell line.

Cuture medium: Dulbecco Minimal Eagle Medium (DMEM).

To 100 mls DMEM add; 20 mls foetal bovine serum

2 mls penicillin/streptomycin (5000 units/ml)

1 ml glutamine (200 mM)

1 ml non-essential amino acids

Amphotericin B (Fungizone) (250 μg/ml).

B.2 Miscellaneous tissue culture reagents

Trypsin

0.25% trypsin in EDTA-Versene.

D-mannose

Stock solution; 20% w/v D-mannose in distilled water. Dilute to 1% in TCM for adhesion tests.

Giemsa

5% v/v in phosphate buffer (5.447 g Na_2HPO_4, 4.752 g K_2HPO_4 mix in a mortar, then add 1 g of mix to 2 l distilled water pH 7).

Reagents for mounting adhesion tests

1) Acetone

2) Acetone-Xylene (50/50 v/v)

3) Acetone-Xylene (33/66 v/v)

4) Xylene

5) DePex mountant (BDH).

Pontamine Blue (for Infant Mouse Test for ST)

Pontamine blue 2% w/v in distilled water.

C. Immunoassays

ELISA Solutions

Carbonate Buffer

10.6 g Na_2CO_3 (0.05 M)

0.406 g $MgCl_2.6H_20$ (0.001 M)

2 l distilled water.

Dissolve salts separately. Adjust to pH 9.8.

Diethanolamine buffer

97 mls diethanolamine

800 mls distilled water

0.2 g azide (NaN_3)

100 mg $MgCl_2.6H_2O$.

Make up to 1 l and adjust to pH 9.8 with HCl, store at -4°C in the dark.

Coating Buffer

Prepare fresh for immediate use;

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

800 mls distilled water, adjust to pH 9.6 and made up to 1 l.

PBS-Tween PBS containing Tween-20 (0.5 ml/l).

Substrate for Alkaline Phosphate-conjugated immunoglobulins

Immediately before use dissolve para-nitrophenol phosphate in diethanolamine buffer (1 mg/ml).

Stop solution 3 M NaOH (12 g NaOH in 100 mls).

D. Solutions for hybridisation

D.1 Probe preparation

QIAGEN-tip 5 buffers

Buffer A 400 mM NaCl, 50 mM MOPS, 15% ethanol pH 7.0

Buffer B 750 mM NaCl, 50 mM MOPS, 15% ethanol pH 7.0

Buffer F 1500 mM NaCl, 50 mM MOPS, 15% ethanol pH 7.5

10 x MOPS buffer 0.2 M 3-[Morpholino]-propane-sulphonic acid

0.05 M sodium acetate, pH 7.0

0.01 M EDTA

D.2 Solutions for preparation of colony blots

1) 10% Sodium dodecyl sulphate (see D.3)
2) Alkali denaturing solution (0.5 M NaOH, 1.5M NaCl)

   1 g NaOH, 4.4 g NaCl in 50 mls distilled water.
3) Neutralising solution (1.5 M NaCl, 0.5 M Tris, pH 8)

   43.8 g NaCl, 30.3 g Tris in 400 mls distilled water, adjust pH using HCl and make up to 500 mls.
4) 2x SSPE (see D.3 below). Dilute 20x SSPE, 1/10.

D.3 Stock solutions

20x SSC 175.3 g NaCl

88.2 g Na_3 citrate in 1 l distilled water, pH 7.0.

20x SSPE 78 g NaCl

15.6 g $NaH_2PO_4.2H_20$

3.7 g EDTA in 1 l distilled water, pH7.4

10% SDS 50g sodium dodecyl sulphate dissolved in 400 mls distilled water, make up to 500 mls.

D.4 Hybridisation buffers

DIG hybridisation buffer (used for CT at high stringency, VT polynucleotide and GM1 oligonucleotide)

5x SSC

1% blocking reagent (BCL)

0.1% sodium-lauroylsarcosine

0.02% SDS.

CT hybridisation buffer (low stringency)

75 mls Fluka formamide

30 mls *50x Denhardt's solution* 1% (w/v) BSA (bovine serum albumin), 1% (w/v) Ficoll ™ (Pharmacia), 1% (w/v) PVP (polyvinylpyrollidone)

30 mls 20x SSPE

1.5 ml 20% SDS

115.5 mls distilled water.

ST hybridisation buffer

6 x SSC

0.1% ficoll

1% BSA

0.1% polyvinylpyrolidone (PVP)

1 mM EDTA.

SNAP-LT and SNAP-ST hybridisation buffer

5x SSC

1% SDS

0.5% BSA

0.5% polyvinylpyrolidone (PVP).

D.5 Immunological detection of hybrids

Detection of Digoxigenin labelled probes

Buffer 1 100 mM Maleic acid

150 mM NaCl pH 7.5.

Buffer 2 1% blocking reagent in buffer 1 (dissolve 50 - 70oC 30 min or make a 10X solution, autoclave and store in fridge).

Buffer 3 100 mM Tris-HCl

100 mM NaCl, 50 mM MgCl2 pH 9.5.

Buffer 4 10 mM Tris-HCl

1 mM EDTA, pH 8.

Colour solution 45 µl Nitroblue tetrazolium (NBT)

35 µl 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)

10 mls buffer 3.

Detection of SNAP probes

66 µl NBT

50 µl BCIP

15 mls Alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5).

D.6 Southern blot analysis

Solutions for preparation of genomic DNA

10 mg/ml proteinase K (Sigma).

5 M NaCl.

10% CTAB (Sigma) in 0.7 M NaCl dissolve at 65°C.

24: 1 chloroform/isoamyl alcohol.

25 g: 24: 1 phenol/cholorform/isoamyl alcohol.

Isopropanol.

70% ethanol.

TE/RNase (5 µl 10 mg/ml RNase in 1 ml TE).

Restriction endonucleases (Gibco-BRL)

*Eco*R1	Buffer R3
*Hin*dII	Buffer R2
*Bgl*II	Buffer R3
*Pst*1	Buffer R2
*Xba*I	Buffer R2

Buffer R2 - 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 50 mM NaCl.

Buffer R3 - 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 100 mM NaCl.

Solutions for Southern blotting

Depurination solution	0.25 M HCl.
Denaturing solution	0.5 M NaOH, 1.5 M NaCl.
Neutralising solution	0.5 M Tris, 1.5 M NaCl, pH 7.4.
Blotting solution	6x SSC

D.7 Solutions for Plasmid preparations

Solution 1	0.05 M Glucose, 0.01 M EDTA, 0.025 M Tris-HCl pH 8
Solution 2	0.2 N NaOH, 1% SDS
Solution 3	3 M Sodium acetate, pH 4.8
Solution 4	0.1 M Na acetate, 0.05 M Tris-HCl, pH 8

E. Protein and LPS analysis

Lysis buffer protein analysis

8% distilled water

20% glycerol

2% mercaptoethanol

0.8 g SDS

70% 0.5M Tris-HCl, pH 6.8

Lysis buffer incorporating bromophenol blue

5 mls lysis buffer (above)

5 mls distilled water

10 µg bromophenol blue.

Determination of protein using Lowry procedure

Lowry solution A	2% (w/v) Na_2CO_3 in 0.1 M NaOH
Lowry solution B	0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) Na,K Tartrate
Lowry solution C	Mix 50 mls solution A and 1ml solution B.
Lowry solution D	1N Folin-Ciocalteau reagent (BDH)

Calibration curve for protein concentration

1 ml of protein standard containing 1 mg BSA, was mixed with 4 mls distilled water.

Standards were prepared by mixing the following:-

Protein µg ml	Diluted Standard (ml)	Water(ml)
Blank	0.00	1.00
50	0.25	0.75
100	0.50	0.50
150	0.75	0.25
200	1.00	0.00

5 mls of solution C was added, mixed thoroughly and left for 10 min, followed by the addition of 0.5 ml solution D, mixed and left for 30 min. Optical density was read at 500nm. For calibration curve, plot OD_{500} on 'Y' axis against µg Protein on 'X' axis.

Calculation for 30 µg protein in a final solubilization buffer volume of 30 µl.

$$\text{Volume of protein preparation} = \frac{30}{\text{Protein conc } \mu g/\mu l}$$

Volume of solubilizer = 60 - (the above volume x 2)

This gives you twice the volume you require.

LPS Solubilization buffer

10% (v/v) glycerol

5% (v/v) 2-mercaptoethanol

3% (w/v) SDS

62.5 mM Tris-HCl pH 6.8

0.01% (w/v) bromophenol blue

F. Gel buffers and stains

F.1 Agarose Gel buffers

Tris acetate buffer 40 mM Tris

5 mM sodium acetate

1 mM EDTA, pH 7.9 with glacial acetic acid.

Tris borate buffer 90 mM Tris

2 mM EDTA

90 mM Boric acid.

F.2 SDS-Polyacrylamide gel solutions

Lower gel buffer (LGB)	Tris-HCl 1.5 M pH 8.8, SDS 0.4%
Upper gel buffer (UGB)	Tris-HCl 0.5 M pH 6.8, SDS 0.4%
Acrylamide	30% (w/v) Acrylamide, 0.8% (w/v) bis-acrylamide.
Ammonium persulphate (APS)	10% (w/v) make fresh in distilled water.
Tetramethylethlenediamine	use undiluted stock (TEMED).
Running buffer	6.06 g Tris, 28.8 g Glycine, 2 g SDS, make up to 2 l with distilled water.

SDS-PAGE Gel

Resolving gel was 5% acrylamide (10 mls LGB, 13.4 mls distilled water, 16.6 mls acrylamide, 120 µl APS, 10 µl TEMED).

Stacking gel was 5% acrylamide (2.5 mls UGB, 6 mls distilled water, 1.5 mls acrylamide, 30 µl APS, 10 µl TEMED).

F.3 Stains

Ethidium bromide for staining DNA gels

Stock solution - 700 µg/ml in TE buffer

For use dilute 200 µl in 250 ml distilled water (0.56 µg/ml).

Bromophenol blue electrophoresis marker

60% sucrose (w/v), 0.25% bromophenol blue (w/v) in TE buffer.

Orange G electrophoresis marker

60% sucrose (w/v), 0.25% orange G (w/v) in TE buffer.

Coomassie billiant blue

50% (v/v) Methanol, 5% (v/v) Acetic acid, 0.025% (w/v) Coomassie Brilliant blue

Silver stain

Add 2 mls conc. NH_4OH to 28 mls 0.1 M NaOH. Add 5 mls 20% silver nitrate with agitation, make up to 150 mls with distilled water.

Appendix 2

Strain catalogue

List of V.cholerae non-O1 and V.mimicus by reference number

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0385750	O?		?	Y	23	M
E0385870	O 2		Somalia	Y	23	F
E0385890	O 2		Somalia	Y	4	M
E0385980	5609		Thailand	Y	24	M
E0386510	O?		Middle East	?	?	?
E0387800	O?		Saudi Arabia	Y	?	?
E0387850	O 41		Nepal	Y	49	M
E0388200	O 2		Bangladesh	Y	?	?
E0390530	O 5		India	?	51	M
E0390560	O?		Majorca	Y	56	M
E0390590	O 2		Portugal	Y	?	M
E0390860	5609		Pakistan	Y	<1	M
E0390900	O 34		Malta	Y	77	F
E0391810	O?		India	Y	22	M
E0391930	O?	sea water				
E0393640	O?		Turkey	Y	60	F
E0393810	O 5	blood	?	?	75	F
E0394040	O?	bile	Iran	N	84	M
E0394460	O?		Malta	Y	54	M
E0394570	O?		China	Y	?	M
E0396650	O 27		Spain	Y	25	F
E0396830	O 76		Turkey	Y	25	M
E0397190	OR		India	Y	29	M
E0397980	O?		Spain	Y	1	F
E0397990	O?		Spain	Y	1	F
E0398000	O?		Spain	Y	1	F
E0399340	O?		India	Y	26	F
E0399410	OR		India	Y	56	F
E0399470	O?	prawn				
E0399740	O 22		Majorca	?	50	F
E0399800	O 4		Saudi Arabia	Y	?	M
E0399950	O?		Malta	Y	?	M
E0399960	O?		Spain	Y	50	F

* an asterix after the reference number indicates the strain is a *V.mimicus*, other strains were *V.cholerae*.

[a] site of isolation or environmental source, strains were isolated from faeces unless otherwise stated in this column.

[b] presence or abscence of diarrhoea; Y = diarrhoea present, N =patient did not have diarrhoea, ? =it was not stated whether the patient had diarrhoea or not.

[c] age is stated in years.

[d] M =male; F =female; ? =unknown.

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0400160	O?		Israel	Y	60	M
E0400530	O?		Portugal	Y	43	F
E0400600	O?		Turkey	Y	31	M
E0400790	OR	pond water				
E0400800	O?	pond water				
E0400820	OR	pond water				
E0400840	O?		Ibiza	Y	24	F
E0400920	O 54	river				
E0400930	O 76	marina				
E0402020	O?		Tunisia	Y	2	M
E0402080	O?	prawn				
E0402100	O 63	ditch water				
E0402130	O 63	sea water				
E0402131*	O?	sea water				
E0403870	O?		Gambia	Y	?	F
E0404540	O?		Gambia	Y	?	F
E0406200	O?		Hong Kong	?	63	M
E0406310	O 34		Kenya	Y	27	?
E0406320	O 13		Sri Lanka	Y	47	?
E0406700	O?		India	Y	41	F
E0409470	O 5		Bali	Y	?	M
E0410500	O?		Kenya	Y	26	F
E0410510	O 21		Kenya	Y	26	F
E0412310	O?	gull liver				
E0415740	O?		Sri Lanka	Y	2	F
E0415750	O?		Sri Lanka	Y	2	F
E0415760	O?		Kenya	Y	36	M
E0416490	O 46		India	Y	46	M
E0418040	O?		Brazil	Y	47	F
E0418250	O?		Far East	Y	?	M
E0418350	OR		Pakistan	?	?	?
E0418620	O?		India	Y	40	M
E0419620	O?		India	Y	?	F
E0420030	O?		India	Y	?	M
E0420080	O?	river				
E0422560	O?		India	Y	?	F
E0425960	O 5	water				
E0425970	O?	water				
E0427200	O 34		India	?	24	M
E0427510	O 41			Y	26	F
E0428700	O?	water				
E0428710	O 16	water				
E0428780	O 13		India	Y	53	M
E0429510	O?		Tunisia	Y	23	F
E0429560	O?			Y	34	F
E0429930	O 34	river				
E0429940	O?	river				
E0429950	OR	river				
E0429960	OR	river				
E0429970	O?	river				
E0431050	O?		Morocco	?	?	M
E0431490	O?		Morocco	?	?	M
E0431820	O 5		India	Y	56	F
E0432570	O 13	shrimp				
E0432980	OR			Y	48	M
E0433230	O?	prawn				

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0433300	O 2		Tunisia	?	36	M
E0434260	O?		Malta	?	?	F
E0434330	O?		Malta	Y	?	M
E0435040	O?	appendix	Saudi Arabia	?	?	?
E0435630	O?		Morocco	?	60	M
E0436400	O 2		Egypt	Y	19	F
E0436530	OR		Morocco	Y	21	F
E0437240	OR		Spain	?	?	F
E0437560	O?	shrimp				
E0437580	O?	prawn				
E0437600	O 58	prawn				
E0437610	O?	prawn				
E0437650	O?	prawn				
E0437730	O?		India	Y	55	M
E0438120	O 58		Tenerife	Y	52	F
E0438430	O?		India	Y	?	M
E0438540	O 11	blood	Arab Emirates	?	?	?
E0438670	O?		Africa	?	40	M
E0438680	O?		Africa	?	40	M
E0438940	O?	prawn				
E0439660	O 6		Spain	Y	8	M
E0440910	O?	prawn	Thailand			
E0440920	O?	prawn	Thailand			
E0440940	O?	prawn	Nigeria			
E0441180	O 37		Gambia	Y	20	M
E0441640	O?		Majorca	Y	38	F
E0442150	O?	prawn				
E0442160	O 3	prawn				
E0442400	O 31		Kenya	Y	30	F
E0442530	O?		India	Y	60	F
E0443160	O?		Egypt	Y	58	F
E0444550	O?	mussells				
E0444700	O?	cockles				
E0445130	O 41	food				
E0445140	O?	food				
E0445150	O?	food				
E0445510	O 4	river				
E0447000	O 39		Spain	Y	26	M
E0449290	O?		Egypt	Y	22	F
E0449430	O?	food				
E0449460	O 41	food				
E0452420	O 37		Kenya	Y	?	M
E0453060	O 62	mussells				
E0453120	O 41	prawn				
E0453180	O?	marina				
E0453570	O 31		Kenya	Y	63	M
E0455110	O?		Sri Lanka	Y	28	F
E0455180	O 19	prawn				
E0455190	OR	prawn				
E0455200	O?	prawn				
E0455210*	O 14	prawn				
E0455220	O?	prawn				
E0455230	O?	prawn				
E0456250	OR		Bahamas	Y	29	F
E0456960	O 34		Thailand	Y	62	M
E0458810	O 37		Kenya	Y	67	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0458820	O?		Pakistan	Y	37	M
E0458830	O 5		Burma	Y	52	M
E0460070	O?	shellfish				
E0461460	OR	shellfish				
E0461510	O?	shellfish				
E0461520	OR	shellfish				
E0461530	O?	shellfish				
E0461540	O 49	shellfish				
E0462340	O 43	prawn				
E0462350	O?	prawn				
E0462370	OR	prawn				
E0462380	O?	prawn				
E0462500	O 9	prawn				
E0462510	O?	prawn				
E0462720	OR	prawn				
E0465041	OR	prawn				
E0465051	5609	prawn				
E0465060	O?	prawn				
E0465070	O?	prawn				
E0466840	O?		Madeira	Y	24	M
E0467630	O 63	prawn				
E0467640	O 44	prawn				
E0467970	O 5		Thailand			
E0467980	OR		Thailand			
E0467990	O 39		Thailand			
E0468000	O?		Thailand			
E0468010	O?		Thailand			
E0468020	O 56		Thailand			
E0468030	OR		Thailand			
E0468040	O 11		Thailand			
E0468050	O?		Thailand			
E0468060	O?		Thailand			
E0468080	O 34		Thailand			
E0468090	OR		Thailand			
E0468130	O?		Thailand			
E0468140	O?		Thailand			
E0468150	O?		Thailand			
E0468160	O?		Thailand			
E0468170	O 11		Thailand			
E0468180	OR		Thailand			
E0468190	O 34		Thailand			
E0468200	OR		Thailand			
E0468210	OR		Thailand			
E0468220	O 25		Thailand			
E0468230	O 67		Thailand			
E0468240	O?		Thailand			
E0468250	O?		Thailand			
E0468260	O?		Thailand			
E0468270	O?		Thailand			
E0468280	O?		Thailand			
E0468290	O 3		Thailand			
E0468300	OR		Thailand			
E0468310	O 2		Thailand			
E0468320	O 46		Thailand			
E0468330	O 20		Thailand			
E0468340	O 21		Thailand			

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0468350	O?	water	Thailand			
E0468360	O?	water	Thailand			
E0468370	O 68	water	Thailand			
E0468380	O 62	food	Thailand			
E0468390	OR	food	Thailand			
E0468400	O 13		Thailand			
E0468410	OR		Thailand			
E0468420	O?		Thailand			
E0468430	OR		Thailand			
E0468440	O 5		Thialand			
E0468450	O?		Thailand			
E0468460	O 76		Thailand			
E0468490	OR		Thailand			
E0468820	OR		Italy			
E0469050	O?		Italy			
E0469060	O 16		Italy			
E0469490	O 6		India	Y	19	M
E0469890	OR		Kenya	Y	?	F
E0470060	O?		Kenya	?	32	F
E0470580	O 39	prawn				
E0470590	O 61	prawn				
E0470600	O?	prawn				
E0470610	O?	prawn				
E0470620*	O 34	prawn				
E0470630	OR	prawn				
E0470640	O?	prawn				
E0470650	O?	prawn				
E0470660	O?	prawn				
E0470930	O?	prawn				
E0471640*	O?	prawn				
E0471660	O?	prawn				
E0471670	O?	prawn				
E0471680	O?	prawn				
E0471690	O?	prawn				
E0471730	O 19	prawn				
E0471740	O 19	prawn				
E0471750	O 19	prawn				
E0471760	O 19	prawn				
E0471770	O 19	prawn				
E0471780	O 19	prawn				
E0471790	O?	prawn				
E0471800	O 19	prawn				
E0472610	O?		Thailand	Y	39	F
E0472700	5609	prawn				
E0472710	5609	prawn				
E0472720	O 19	prawn				
E0472730	5609	prawn				
E0472740	O 76	prawn				
E0472750	5609	prawn				
E0472760	O?	prawn				
E0472770	O?	prawn				
E0472780	O 19	prawn				
E0472790	O 19	prawn				
E0475340	OR		Venezuela			
E0475350	OR		Venezuela			
E0475610	O 5		S.E. Asia	?	51	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0475710	O?		Minorca	Y	18	M
E0478310*	O 39	prawn				
E0478360	O?	prawn				
E0478370	O?	prawn				
E0478380	O?	prawn				
E0479570	O?		Turkey	Y	34	M
E0480650	O 6		Sri Lanka	Y	25	F
E0482230	O 24		Turkey	Y	?	M
E0482380	O 8	blood	Jordan	?	?	?
E0485990	OR		Greece	Y	43	F
E0486410	O?	prawn				
E0486450	O?	prawn				
E0486460	O 9	prawn				
E0487490	O 24		Pakistan	Y	42	M
E0487700	O?		?	?	?	?
E0487840	O?		India	Y	50	F
E0488620	O?		Tunisia	Y	47	F
E0488820	O 5		Phillipines	Y	38	M
E0488950	O 8		Cyprus	Y	22	M
E0489170	O 2		Tunisia	Y	36	F
E0489270	O 2		India	Y	?	M
E0489280	O 13		Thailand	Y	43	M
E0489470	O?		Tunisia	Y	47	F
E0489480	O?		Barbados	Y	26	F
E0489590	O 34		Portugal	Y	28	?
E0489830	O?		India	Y	20	M
E0490320	O 4	prawn				
E0490360	O?	prawn				
E0490810	O?		Turkey	Y	59	M
E0491080	O 14		Malta	Y	52	F
E0492590	O?		India	Y	49	M
E0495710	O 76	prawn				
E0495730	OR	prawn				
E0497030	O?		India	Y	29	M
E0500530	O 24		Central America	Y	49	M
E0501650	O?		Kenya	Y	65	F
E0501770	O 13		Chile	Y	43	M
E0517030	O?	prawn				
E0517040	O?	prawn				
E0517050	O?	prawn				
E0517060	O 3	prawn				
E0517070	O?	prawn				
E0517080	O?	prawn				
E0517090	O 2	prawn				
E0517100	OR	prawn				
E0517110	O 14	prawn				
E0517120	O 14	prawn				
E0517130	OR	prawn				
E0517140	OR	prawn				
E0521030	O 9	prawn				
E0521040*	O?	prawn				
E0521600	OR	cockles				
E0521610	O?	cockles				
E0521620	OR	cockles				
E0521630	OR	cockles				
E0523450	O?	cockles				

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0523460	OR	cockles				
E0523470	O?	cockles				
E0523480	O?	cockles				
E0523490	O?	cockles				
E0523660	O?	cockles				
E0523670	OR	cockles				
E0523680	O?	cockles				
E0523690	O?	cockles				
E0523700	O?	cockles				
E0523710	OR	water				
E0523720	OR	water				
E0523910	O?	cockles				
E0523920	O?	cockles				
E0523930	O?	cockles				
E0523940	O?	cockles				
E0523950	O?	cockles				
E0523960	O?	cockles				
E0523970	O?	cockles				
E0523980	O?	cockles				
E0523990	O?	water				
E0524000	O?	water				
E0524010	O?	water				
E0524020	OR	water				
E0524030	O?	water				
E0525180	O?		India	Y	?	M
E0525520	O 18		Kenya	Y	43	F
E0526480	O 11		Kenya	?	18	M
E0527700*	O 39	prawn				
E0527710	O 16	prawn				
E0527721	O?	prawn				
E0527730	O?	prawn				
E0527740	O 3	prawn				
E0527750	O?	prawn				
E0527760	O?	prawn				
E0527770	O?	prawn				
E0527780	O 76	prawn				
E0527790	O 18	prawn				
E0528650	O?	shellfish				
E0528660	O?	shellfish				
E0528670	O 58	shellfish				
E0528680	O 13	shellfish				
E0528690*	O?	shellfish				
E0528910	O?		Nepal	Y	56	F
E0532880	O?	seafood				
E0532900	O 81	seafood				
E0535170	O?		Egypt	Y	60	M
E0535720	O?		India	Y	21	F
E0538010	O?		India	Y	24	M
E0538890	O 37		Singapore	Y	43	F
E0538900	O?		Thailand	Y	62	M
E0538980	O?		Thailand	Y	48	F
E0540410	O?		Tunisia	Y	27	M
E0540480	O?		Egypt	Y	53	M
E0541170	O 5		India	Y	32	M
E0541220*	O 20		Phillipines	Y	17	M
E0541360	O?		Tunisia	Y	21	F

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0542340	OR		Tunisia	Y	57	M
E0543110	O?		Spain	Y	36	F
E0543120	OR		Portugal	?	46	F
E0543800	O 37		India	Y	27	M
E0543810	O 37		Tunisia	Y	15	F
E0543820	O?		Spain	Y	48	M
E0543830	5609			?	?	?
E0544020	O 24		India	Y	52	M
E0544180	O 37		Tunisia	Y	19	M
E0544370	O 4		Tunisia	Y	5	F
E0544680	O?		Egypt	Y	34	F
E0545010	O 34		Kenya	Y	37	F
E0545890	O 13		India	Y	53	F
E0546220	O 24		Malta	Y	48	M
E0546650	O 2		Tunisia	Y	22	F
E0546660	OR		Tunisia	Y	22	F
E0547580	OR		Tunisia	Y	46	M
E0548040	OR		Tunisia	Y	18	F
E0548200	OR		Tunisia	Y	?	M
E0548210	O?		India	Y	29	M
E0548710	O 62	water	Italy			
E0548730	O 6	water	Italy			
E0549140	OR	sewage	Italy			
E0549150	OR	sewage	Italy			
E0549160	OR	sewage	Italy			
E0549170	OR	sewage	Italy			
E0549180	OR	sewage	Italy			
E0549190	OR	sewage	Italy			
E0549200	O?	sewage	Italy			
E0549220	O?	sewage	Italy			
E0549230	O?	sewage	Italy			
E0549240	O 70	sewage	Italy			
E0549260	OR	water	Italy			
E0549270	OR	water	Italy			
E0549280	O?	water	Italy			
E0549290	OR	water	Italy			
E0549300	OR	water	Italy			
E0549310	O 14	water	Italy			
E0549320	OR	water	Italy			
E0549330	OR	water	Italy			
E0549390	O?	water	Italy			
E0549400	O?	water	Italy			
E0549410	O?	water	Italy			
E0549420	O?	water	Italy			
E0549440	OR	water	Italy			
E0549460	OR	water	Italy			
E0549470	O 14	water	Italy			
E0549480	OR	water	Italy			
E0549490	OR	water	Italy			
E0549500	O 62	water	Italy			
E0549510	O 79	water	Italy			
E0549520	O 70	water	Italy			
E0549530	O?	river	Italy			
E0549540	O 25	river	Italy			
E0549550	OR	river	Italy			
E0549560	O 25	river	Italy			

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0549570	O?	river	Italy			
E0549590	O?	river	Italy			
E0549600	O?	sewage	Italy			
E0549610	O 32	sewage	Italy			
E0549630	O 64	sewage	Italy			
E0549640	OR	sewage	Italy			
E0549650	O 32	sewage	Italy			
E0549660	OR	water	Italy			
E0549670	OR	water	Italy			
E0549680	O?	water	Italy			
E0549690	O 34	water	Italy			
E0549700	O?	water	Italy			
E0549710	OR	water	Italy			
E0549720	O 41	water	Italy			
E0549740	O?	water	Italy			
E0549830	O 6	water	Italy			
E0549880	O 4	water	Italy			
E0549890	O?	water	Italy			
E0549900	O?	water	Italy			
E0549930	OR	water	Italy			
E0549940	O?	water	Italy			
E0549960	OR	water	Italy			
E0549970	O?	water	Italy			
E0549990	O?	water	Italy			
E0550000	OR	water	Italy			
E0550010	OR	water	Italy			
E0550070	OR		India	Y	21	F
E0550160	OR		Tenerife	Y	38	F
E0550230	OR		Malta	Y	?	F
E0551210	O?		India	Y	20	F
E0551220	O?		India	Y	?	M
E0551300	O 42	blood	India	?	?	?
E0551400	O 13		India	Y	35	F
E0551770	O?		Bali	Y	39	F
E0551780	O 58		Turkey	Y	68	F
E0552690	O?		India	Y	24	M
E0553120*	O?	prawn	Malaysia			
E0553140	O?		Morocco	Y	?	F
E0554130	O?		Egypt	?	23	M
E0554140	O?	?wound			46	M
E0554150	O?	?wound			46	M
E0554160	O?	?wound			46	M
E0555100	O?		Spain	Y	23	F
E0556620	OR		India	Y	66	M
E0558790	O?		Egypt	Y	22	M
E0558820	O?	fish tank				
E0561090*	O?	shrimp	U.S.A.			
E0565660	O?		Maldives	Y	41	F
E0568720	O 9	prawn				
E0570240	O?	prawn				
E0570250	O?	prawn				
E0570260	O?	prawn				
E0571210	O?		Bali	?	36	F
E0572000	O?	prawn				
E0572010*	O?	prawn				
E0572020*	O?	prawn				

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0572030	O?	prawn				
E0572040*	O?	prawn				
E0572050*	O?	prawn				
E0572060	O 32	prawn				
E0572070	O?	prawn				
E0572080	O 79	prawn				
E0572090	O?	prawn				
E0572100*	O?	prawn				
E0572280	O?		Kenya	?	?	F
E0572760	O 79	prawn				
E0575890	O?	prawn				
E0575900	O?	prawn	India			
E0575910*	O?	prawn	India			
E0576910	OR		Peru	Y	2	F
E0576930	OR		Peru	?	1	F
E0576990	OR		Peru	?	<1	M
E0577100	OR		Peru	Y	1	F
E0577120	O?		Peru	Y	2	M
E0578560	O?	prawn	India			
E0578570	O 67	prawn	India			
E0578740	O 2		Tenerife	Y	24	M
E0580170	O 13		India	?	31	F
E0580500	O?		India	Y	27	F
E0580510	O 43	prawn	Malaysia			
E0580520*	O?	prawn	Malaysia			
E0580600	O?		Iraq	Y	30	M
E0583100	O?		S.E. Asia	Y	?	M
E0583450*	O?	prawn				
E0583460*	O?	prawn				
E0583470*	O?	prawn				
E0584120	O?		Pakistan	Y	35	M
E0584630	O?		Nepal	Y	20	F
E0585220	O 2		Iraq	Y	45	M
E0585460	O?		India	Y	13	F
E0588150	OR		Tunisia	Y	26	M
E0588470	O?	shrimp	S.E. Asia			
E0588480	OR	shrimp	S.E. Asia			
E0588590	O?	shrimp	S.E. Asia			
E0588600	O 49	shrimp	S.E. Asia			
E0588620	O 49	shrimp	S.E. Asia			
E0588630	O?	shrimp	S.E. Asia			
E0588640	O 49	shrimp	S.E. Asia			
E0588890*	O 6	prawn	Malaysia			
E0588920*	O 6	prawn	Malaysia			
E0588940*	O?	prawn	Malaysia			
E0589340	O?		Tunisia	?	4	M
E0589730*	O 41	prawn	Malaysia			
E0590530	O 22		Dominica	Y	23	F
E0591020	OR		Australia	Y	42	F
E0591440	OR	shrimp				
E0591450	OR	shrimp				
E0591460	OR	shrimp				
E0591461	OR	shrimp				
E0591720	O?		Tunisia	Y	37	M
E0593240	O 11		India	Y	30	F
E0594190	O?		Spain	Y	10	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0594240	O?		Tunisia	Y	24	M
E0594250	O?		Majorca	Y	26	M
E0595200*	O?	prawn	Malaysia			
E0595210*	O?	prawn	Malaysia			
E0595220*	O?	prawn	Malaysia			
E0595230	O?	prawn	Malaysia			
E0596060	O?		Malta	Y	30	F
E0596800	O 13		India	Y	25	F
E0597160	O?		Tunisia	Y	49	F
E0598350	O 13		Far East	Y	32	F
E0598360	OR		Egypt	Y	?	F
E0599130	OR		Caribbean	Y	45	M
E0601500	O 34		Egypt	Y	50	F
E0602560	O?		Pakistan	Y	46	F
E0602640	O?	prawn				
E0602790	OR		Canary Islands	Y	60	M
E0603900	O 39	shrimp				
E0603910	O?	shrimp				
E0603920	O 39	shrimp				
E0603930	O?	shrimp				
E0604220	O?		Pakistan	Y	35	M
E0605440	OR		Ibiza	Y	52	M
E0605940	O?	shrimp				
E0608900*	O 71	prawn				
E0608910*	O?	prawn				
E0608920*	O?	prawn				
E0609460	O 61		India	Y	55	M
E0612460	O?	shrimp				
E0612470	O?	shrimp				
E0616380*	O?	prawn				
E0616450	O?	sea water				
E0616460	O?	sea water				
E0616470	O?	sea water				
E0616480	O?	sea water				
E0616490	O?	sea water				
E0616500	O 63	sea water				
E0616510	O?	sea water				
E0617300	O?	prawn				
E0617800	O?	prawn				
E0617810	O?	prawn				
E0617820	O?	prawn				
E0617830	O?	prawn				
E0617840	O?	prawn				
E0617850	O?	prawn				
E0617860	O?	prawn				
E0618470	O?	prawn				
E0618480	O?	prawn				
E0619460	O?	prawn				
E0619470	O?	prawn				
E0619480	O?	prawn				
E0620810	O?	prawn				
E0621250	O?	prawn				
E0621260	O?	prawn				
E0621270	O 19	prawn				
E0621280	O?	prawn				
E0623100	O 61	sea water	Barbados			

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0625890	O?		Thailand	Y	65	F
E0626030	O?		India	?	53	M
E0626350	O?		Kenya	?	60	F
E0626360	O?		Kenya	Y	60	M
E0626750	O 2		Thailand	Y	49	M
E0629040	O?		Australia	?	?	M
E0630990	O 10		Kenya	Y	28	F
E0634530*	O?		Thailand	Y	25	F
E0635850	O 2		India	Y	26	F
E0636240	O 10		Kenya	Y	33	F
E0637300	O?		India	Y	32	M
E0638070	O 14		Kenya	?	?	F
E0638900	O?		Caribbean	Y	30	M
E0638910	O?		Kenya	Y	20	M
E0641090*	O?	seafood				
E0644110*	O?	seafood				
E0642630	O 38		S. Africa	Y	48	M
E0643660	O 79		Nigeria	Y	29	F
E0644300	O?		Singapore	Y	52	M
E0644630	O?		Cyprus	Y	53	M
E0645020	O?		Kenya	Y	64	F
E0645710	O?		Kenya	Y	25	F
E0646300	O?		Kenya	Y	?	F
E0646920	O 39		Pakistan	Y	?	M
E0647530*	O?	prawn				
E0647540*	O 39	prawn				
E0649950	O 79		Kenya	Y	34	M
E0650940	O?		Kenya	Y	44	F
E0651110	O 9	prawn				
E0651680	O?		India	Y	26	F
E0652490	O?		Kenya	Y	?	F
E0653130	O 2		Morocco	Y	24	F
E0653140	O 2		Morocco	Y	25	M
E0653490	OR		Pakistan	?	20	M
E0653750	O?		St. Lucia	Y	23	F
E0654150	O?		India	Y	23	M
E0654850	O?		Africa	Y	43	M
E0655400	O?		Morocco	Y	44	M
E0656440	O?		India	Y	46	M
E0656730	O?		Turkey	Y	37	F
E0657770	O?		India	Y	41	M
E0657960	O?		Turkey	Y	24	F
E0658420	O?	prawn				
E0658430	O?	prawn				
E0658440	O?	prawn				
E0658450	O?	prawn				
E0658470	O?	prawn				
E0658480	O 9	prawn				
E0658490*	O?	prawn				
E0659760	O?		Kenya	Y	31	M
E0660580	O?		Pakistan	Y	2	M
E0660590	O?		Kenya	Y	35	M
E0660600	O?	carp				
E0660830	O?		Kenya	Y	52	F
E0661830	O?		India	Y	28	M
E0662580	O 81		Kenya	Y	47	F

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0663710	O?		Tunisia	Y	46	M
E0665440	O?		?	Y	19	M
E0666160	O?		Nigeria	?	30	M
E0667360	O?	chicken feed				
E0668100	O 68	food				
E0668110	O?	food				
E0668230	O?		Morocco	Y	58	F
E0668240	O?		Tunisia	Y	41	M
E0668250	O 39		Tunisia	Y	26	F
E0669520	O 56	river				
E0672520	O?		Thailand	Y	25	F
E0672870	OR	river				
E0673430*	O?	prawn				
E0674480	O?		Kenya	Y	28	M
E0675140	O?		Thailand	Y	?	M
E0675320	O?		Kenya	Y	36	F
E0677930	O?		Africa	Y	44	M
E0679740	O?		Kenya	Y	27	M
E0681510	O 11	prawn				
E0681520*	O?	prawn				
E0681920	O 51	ear		?	19	M
E0682890	OR		Thailand	Y	?	M
E0685570	O?		Kenya	Y	20	F
E0685860	OR		Sri Lanka	Y	43	M
E0686080	O?		Kenya	Y	43	F
E0686550	O?	prawn				
E0688830	OR	river				
E0688840	O?	river				
E0688850	OR	river				
E0688860	OR	river				
E0698550	O?	shrimp	Far East			
E0698560	O 81	shrimp	Far East			
E0698570	O 81	shrimp	Far East			
E0698580	O?	shrimp	Far East			
E0698590	O?	shrimp	Far East			
E0700030	O 14		Pakistan	Y	35	M
E0703970	O 37	shrimp	?			
E0703980	O 37	shrimp	?			
E0705870	O 38		Africa	Y	41	M
E0706330	O?		Ghana	Y	?	F
E0706820	O?	shrimp	?			
E0707640	O?	shrimp	?			
E0707890	O?		India	Y	37	M
E0708340	OR		India	Y	27	F
E0709640	O?	prawn	Malaysia			
E0709650*	5609	prawn	Malaysia			
E0709660	OR	prawn	Malaysia			
E0709670*	5609	prawn	Malaysia			
E0709680*	O 43	prawn	Malaysia			
E0709690*	O 43	prawn	Malaysia			
E0709691*	O 43	prawn	Malsysia			
E0709700*	O 43	prawn	Malaysia			
E0709860	OR		India	?	33	F
E0709870	OR		Kenya	Y	43	M
E0709880	O?		Kenya	Y	26	F
E0709890	O?		Kenya	Y	37	F

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0711490	O?		Kenya	Y	22	M
E0711800	O?	sea water				
E0711810	O?	shrimp	Ecuador			
E0711820	O?	shrimp	Ecuador			
E0711830	O 37	shrimp	Ecuador			
E0711840	5609	shrimp	Ecuador			
E0712560	O?		Thailand	Y	36	?
E0713280	O?	river				
E0713290	O 2		Egypt	Y	1	M
E0713300	O 2		Egypt	Y	4	F
E0713310	O 2		Egypt	Y	38	F
E0715670	O?		Kenya	Y	23	F
E0716190	O?		Dominica	Y	66	F
E0716760	OR		Pakistan	?	?	?
E0716770	O?		India	?	27	M
E0717210	O 38		Kenya	Y	40	M
E0718850	O?		India	Y	?	M
E0720240	O?		Kenya	Y	33	F
E0720600	O?		Kenya	?	22	F
E0722010	O?		Kenya	Y	39	F
E0722360	O?	river	Chile			
E0722370	O?	river	Chile			
E0722380	O?	river	Chile			
E0722390	O?	river	Chile			
E0722400	O?	river	Chile			
E0722410	O?	river	Chile			
E0722420	OR	river	Chile			
E0722430	O?	river	Chile			
E0722440	O?	river	Chile			
E0722450	O?	river	Chile			
E0722460	O?	river	Chile			
E0722470	O?	river	Chile			
E0722490	O?	canal water	Chile			
E0722500	O?	canal water	Chile			
E0722510	O?	canal water	Chile			
E0722520	O?	canal water	Chile			
E0722530	O?	water	Chile			
E0722540	O?	river	Chile			
E0722550	O?	river	Chile			
E0722560	O?	swab	Chile			
E0722570	O?	vegetable	Chile			
E0722580	O?	water	Chile			
E0722590	O?	sewage	Chile			
E0725910	O?		Kenya	Y	63	M
E0726980	O?		India	Y	33	M
E0727750	OR		Thailand	?	?	F
E0727920	O 10	shrimp				
E0728710	O?		Tunisia	Y	66	F
E0728720	O?		India	Y	24	M
E0728760	OR		Mexico	Y	?	M
E0729630	O?		Egypt	?	27	M
E0730500	O?		N. Africa	?	46	F
E0730590	O 22		Tunisia	Y	90	M
E0731000	O 38	mussels				
E0731010	OR	mussels				
E0731540	O?		Pakistan	Y	53	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0731550	O?		Malta	Y	46	F
E0731560	O?		Malta	Y	48	M
E0731600	O?		Malta	Y	?	F
E0732750	O?	ear		?	22	F
E0733170	O?		Kenya	Y	58	F
E0733180	OR		Pakistan	?	?	F
E0734670	O 49		India	Y	23	F
E0734680	O?		India	Y	25	F
E0735030	O 2		India	Y	?	M
E0735050	O?		Tunisia	Y	44	F
E0736890	OR		Tunisia	Y	34	M
E0736900	O?		Tunisia	Y	40	M
E0738080	O?		Tunisia	Y	26	M
E0738790	O?		Tunisia	Y	20	M
E0739900	O?		India	Y	20	F
E0739910	O?		India	Y	20	F
E0739920	O 9		Tunisia	?	?	F
E0740300	O?		Greece	?	?	M
E0740960	O?			Y	53	F
E0741400	O?		India	Y	12	?
E0742220	O?		Kenya	Y	29	M
E0743700	OR		India	Y	27	F
E0744370	O?		Kenya	Y	50	M
E0744380	OR		Kenya	Y	30	M
E0744640	O 10		Malta	Y	62	M
E0745240	O?		Kenya	Y	34	F
E0745250	O?			?	?	M
E0745930	O?		Cyprus	Y	59	F
E0746750	O 27		Kenya	Y	26	F
E0746760	OR		Malta	?	66	F
E0747000	O?		Turkey	Y	68	M
E0747120	5609		India	Y	71	F
E0747680	O 2		Kenya	Y	52	F
E0747690	O?		Kenya	Y	31	M
E0748540	OR		Kenya	Y	51	F
E0749910	O 24		Gambia	Y	27	M
E0751660	OR		Africa	Y	53	M
E0751850	OR		Tunisia	Y	30	M
E0753120	OR		Jamaica	Y	45	F
E0755960	O 76			?	?	M
E0756060	OR		Pakistan	Y	?	?
E0756400	OR		Thailand	?	?	M
E0756730	O?		Kenya	?	64	F
E0757640*	O 14	prawn				
E0757650	O?	prawn				
E0757651	O?	prawn				
E0757690	O?	prawn				
E0757710*	O 20	prawn				
E0757730*	O 20	prawn				
E0758010	O?	prawn				
E0758040	O?	prawn				
E0758820	OR	scallops				
E0758830	OR	river				
E0758840	OR	sea water				
E0758841	O?	sea water				
E0759130	OR		Kenya	Y	70	F

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0761570	OR		Kenya	Y	34	F
E0761720	O?		Bali	Y	27	F
E0761730	O?	mussels				
E0763370*	O?	prawn	India			
E0764570*	O 14	prawn	India			
E0767960	O?		Indonesia	Y	43	M
E0771110*	O?	prawn	Bangladesh			
E0774660	OR		U.S.A.	Y	52	M
E0781150	O?		U.S.A.	Y	47	M
E0781160	O 24		Kenya	Y	58	M
E0783840	O 48		Mexico	Y		
E0783850	O 7		Mexico	Y		
E0783860	O 5		Mexico	Y		
E0783870	O?		Mexico	Y		
E0783880	O?		Mexico	Y		
E0783890	O 24		Mexico	Y		
E0786870	O?		Kenya	Y	34	F
E0790380	OR		Kenya	?	42	F
E0790390	O 39		India	Y	7	M
E0790400	O?		India	Y	31	F
E0791240	O?		Kenya	Y	27	F
E0791480	O?		Bali	?	27	M
E0791920	OR		Meditteranean		55	M
E0791930	O 8		Kenya	Y	41	F
E0793210	OR		India	Y	62	M
E0794310	O?		Kenya	Y	24	F
E0794890	OR		Kenya	Y	41	M
E0795470	O?		Kenya	Y	21	F
E0796790	O?		Kenya	Y	63	M
E0797420	OR		Africa	Y	49	F
E0797770	O?		Indonesia	?	51	F
E0798240	O?		Portugal	Y	22	M
E0798580	O?		Kenya	Y	40	F
E0799010	OR		Saudi Arabia		27	F
E0799020	OR		Tenerife	?	41	F
E0799410	O?		Kenya	?	32	F
E0800490	O 9		India	?	58	M
E0801200	O?		India	Y	64	M
E0804190	O 79		Kenya	Y	46	F
E0806710	O?		India	Y	38	M
E0807120	OR		Kenya	Y	54	M
E0808490	O?		Phillipines	Y	32	M
E0812420	O?		Kenya	?	48	F
E0812430	O 26		?	Y	58	M
E0813420	O?		Caribbean	Y	40	M
E0814310	O?		Kenya	Y	30	M
E0814810	O?		Kenya	Y	27	M
E0815380	O 2		Malta	Y	27	M
E0818000	O?		Tunisia	Y	35	M
E0818420	O?		India	Y	25	F
E0818430	O?		Tunisia	Y	8	F
E0819210	O 9		Mediteranean	Y	65	M
E0819260	OR		?	Y	30	F
E0819600	O 3		Pakistan	?	60	M
E0820820	O?		Turkey	Y	22	M
E0822080	O?		?	Y	84	F

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0822100	O?		India	Y	25	M
E0822110	O?		Tunisia	Y	51	F
E0822450*	O?		Caribbean	Y	?	M
E0822460	O?		Kenya	Y	30	M
E0822900	O 51		Tunisia	Y	46	F
E0824060	O?		Kenya	?	3	F
E0824560	O 24		India	Y	19	F
E0825070	O 28		Tunisia	Y	46	F
E0825800	OR		Israel	Y	59	F
E0825820	OR		Tunisia	Y	49	M
E0826210	O?		India	Y	20	M
E0826660	O?		Tunisia	Y	24	F
E0827100	O 79		Tunisia	Y	?	F
E0827680	O?		Kenya	Y	53	M
E0827900	O?		Tunisia	Y	41	F
E0828290	O?	prawn				
E0829250	O?		Tunisia	Y	40	F
E0829470	O?		Portugal	Y	50	F
E0829480	O?		Tunisia	?	30	F
E0830310	O?		Tanzania	Y	47	F
E0830320	O?		Kenya	Y	23	?
E0830960	O?		Malta	Y	53	M
E0831840	O?		India	Y	24	F
E0835300	O?		Senegal	N	5	M
E0836520	O 3		Kenya	Y	26	F
E0837540	O 36	fish tank				
E0837550	O 36	fish tank				
E0837560	O?	fish tank				
E0837830	O 11		Kenya	Y	44	F
E0838570	O?		India	?	30	F
E0839110	O?		Tunisia	Y	69	F
E0839120	O 9		Malta	Y	57	M
E0839540	O?		Malta	Y	59	M
E0842380	O?		Kenya	Y	53	F
E0843670	O 20		Kenya	Y	43	F
E0846760	O?		Kenya	Y	20	M
E0847480	O?		Nepal	Y	44	M
E0849140	O?		Kenya	Y	40	F
E0850550	O 58	pond water				
E0850560	O 58	pond water				
E0850570	O 2	pond water				
E0850580	O?	fish				
E0850590	O?	fish				
E0850600	O 50	fish				
E0850610	O 50	fish				
E0850620	O?	pond water				
E0850630	O?	pond water				
E0850660	O 79	pond water				
E0850670	O 79	pond water				
E0850710	O 58	pond water				
E0850720	O 58	pond water				
E0850760	O 2	pond water				
E0853930	O 3		India	?	62	F
E0859150	OR		Kenya	Y	40	M
E0859420	O?		Kenya	Y	67	F
E0859430	O139		?	Y	?	?

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0861310	O?		Mexico	Y		
E0861320	O?		Mexico	Y		
E0861330	OR		Mexico	Y		
E0861340	O?		Mexico	Y		
E0861350	OR		Mexico	Y		
E0862520	O?		Kenya	Y	?	?
E0862700	O139		Bangladesh	Y	36	M
E0863080	OR		Kenya	Y	54	M
E0865290	O?		?	?	30	F
E0867320	O?		Kenya	Y	74	M
E0867670	O?		Egypt	Y	26	F
E0867860	O?		Bangladesh	Y	32	M
E0869710	OR		S. Africa	Y	55	F
E0869990	O 9		Kenya	Y	28	F
E0870000	O 9		Kenya	Y	28	F
E0871850	O 46		India	Y	30	M
E0871860	OR		Kenya	Y	31	M
E0871870	OR	blood	Caribbean	?		
E0871880	O?		Caribbean	?		
E0871890	OR	spring	Caribbean			
E0871900	O?	spring	Caribbean			
E0871910	OR	spring	Caribbean			
E0871920	OR	spring	Caribbean			
E0871930	OR	spring	Caribbean			
E0871940	OR	spring	Caribbean			
E0871950	OR	pond water	Caribbean			
E0871960	O?	water	Caribbean			
E0871970	OR	water	Caribbean			
E0871980	OR	water	Caribbean			
E0871990	OR	water	Caribbean			
E0872000	O?		Caribbean	?		M
E0872010	OR	water	Caribbean			
E0872020	O 2	water	Caribbean			
E0872030	OR	water	Caribbean			
E0875090	O139		India	Y	68	?
E0875650	O?		Kenya	Y	31	M
E0875670	O?		Thailand	Y	23	M
E0875680	O?		?	?	36	F
E0877390	O?	blood	Saudi Arabia	?	29	F
E0877560	O 37		Turkey	Y	33	M
E0877980	O 65		Dominica	Y	28	M
E0879540	O139		India	Y	25	F
E0881060	O?		Tunisia	Y	?	M
E0881310	O139		India	Y	19	M
E0881610	O?		Kenya	?	60	F
E0882660	O?		India	?	25	F
E0883660	OR		Tunisia	Y	?	M
E0883830	O76		Egypt	Y	47	M
E0884100	O 11		Far East	Y	50	M
E0885010	OR		Tunisia	?	29	F
E0885550	OR		Tunisia	Y	61	M
E0886040	OR		Bulgaria	?	47	M
E0886590	O?		Malta	?	44	F
E0886600	O?		Malta	Y	44	F
E0886980	O?		Majorca	Y	?	F
E0890050	OR		Dominica	Y	?	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0890060	OR		?	Y	22	F
E0890070	O139		India	Y	30	M
E0890240	OR		Tunisia	Y	30	M
E0893320	O?		India	Y	45	F
E0893590	OR		Pakistan	Y	46	M
E0893880	OR		Tunisia	Y	41	F
E0894160	O?		India	Y	2	F
E0894660	O?		Gambia	Y	35	F
E0895160	OR		Thailand	Y	16	F
E0895360	O?		Tunisia	Y	47	F
E0895370	O?		Kenya	Y	?	F
E0896190	O?		Thailand	Y	47	F
E0896730	O?		India	Y	4	F
E0896920	O?		Egypt	Y	23	M
E0896930	O?		Tunisia	Y	54	F
E0897550	O 14		India	Y	23	M
E0898030	O?		Tunisia	Y	?	M
E0898590	O 24		Morocco	?	?	?
E0898600	O?		?	Y	68	F
E0899080	O?		Tunisia	Y	60	F
E0900590	O?		Japan	Y	53	M
E0900670	O139		Pakistan	Y		
E0900700	O139		Pakistan	Y		
E0900701	O139		Pakistan	Y		
E0901300	O?		Pakistan	Y	23	M
E0901660	O?		Mexico			
E0901670	O?		Mexico			
E0901680	O?		Mexico			
E0901690	O?		Mexico			
E0901700	O?		Mexico			
E0901710	O?		Mexico			
E0901711	O?		Mexico			
E0901720	O?		Mexico			
E0901730	O?		Mexico			
E0901740	O?		Mexico			
E0901750	O?		Mexico			
E0901751	O?		Mexico			
E0901760	O?		Mexico			
E0901770	OR		Mexico			
E0902510	O139		Thailand	Y	25	F
E0902620	O?		Greece	Y	1	F
E0902920	O?		India	Y	19	M
E0905580	5609		Tunisia	?	?	?
E0905590	O?		Tunisia	Y	25	M
E0905900	O?		Far East	Y	52	F
E0906290	O?		Dominica	?	25	F
E0906450	O?		India	Y	21	F
E0906550	O 38		India	Y	?	F
E0906800	O?		Tunisia	Y	41	M
E0907270	O?		Bali	Y	45	F
E0907280	O?		Hong Kong	Y	35	M
E0908110	O?		Mexico	Y	?	?
E0909760	O 51		Tunisia	Y	44	M
E0909970	O?		E. Africa	Y	74	F
E0910220	O 58		?	?	22	F
E0910560	O?	shrimp				

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0910910	O?		S.E. Asia	?	68	F
E0910960	O?		?	Y	38	M
E0911730	O?		Gambia	Y	47	F
E0913350	O?		Kenya	Y	27	F
E0913360	O?		Kenya	Y	27	M
E0914060	O?		Kenya	Y	52	F
E0917230	O?		?	?	23	F
E0918440	O 66		Kenya	Y	25	F
E0918450	5609		India	Y	33	M
E0918900	O 35		Bali	Y	26	F
E0919260	O?	wound	?	?	?	M
E0923140	O?		Gambia	?	20	F
E0926260	O 49		Kenya	Y	39	F
E0926600	O139		Bangladesh	Y	54	M
E0928210	O 9		Sri Lanka	Y	45	M
E0930630	O 38		Peru	?	1	F
E0932340	O 29		India	Y	53	M
E0933070	O 66		Kenya	Y	47	F
E0933260	O 27		India	Y	38	F
E0933430	5609		Middle East	Y	61	F
E0934720	O?	king prawn	Thailand			
E0934730	O?	red mullet	Thailand			
E0935980	O?		Kenya	Y	73	F
E0936200	O?		Kenya	Y	61	F
E0936680	O?		Sri Lanka	Y	?	F
E0938110	O 37		?	?	?	M
E0938180	O?		Kenya	Y	55	F
E0938190	O?	shrimp				
E0939070	O 18		India	Y	38	M
E0940650	O 57		Kenya	Y	26	M
E0941090	O?		Saudi Arabia	Y	5	F
E0941100	O?		India	Y	23	M
E0941110	O?		Bali	Y	88	M
E0943180	O?		India	Y	40	F
E0943830	OR	blood		?	78	M
E0944950	O?		?	Y	1	?
E0948750	O?		Kenya	Y	23	F
E0949020	O?		Oman	?	?	M
E0949030	O?		India	Y	33	M
E0949760	O?		India	Y	64	M
E0951350	O?		?	?	62	F
E0954220	O?		Kenya	Y	38	M
E0954230	O?		Singapore	Y	48	F
E0955910	O?		Pakistan	Y	1	M
E0957320	O?		Kenya	Y	49	M
E0959510	O?	shrimp				
E0961300	O?		Pakistan	Y	?	F
E0964380	O?		Kenya	Y	26	F
E0965140	O 9		Kenya	Y	62	F
E0968040	O 24		India	Y	19	F
E0968320	O 9		Morocco	Y	64	F
E0968600	O?		Sri Lanka	Y	31	F
E0968930	O?		Tunisia	Y	25	F
EO969040	O?		Tunisia	Y	?	F
E0969610	O?		India	Y	37	M
E0970800	O?		Kenya	Y	48	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0971960	O?		Dominica	Y	22	M
E0972150	O 27		Pakistan	Y	40	M
E0973520	O 6		Majorca	Y	39	F
E0973600	O?		Turkey	Y	55	M
E0973980	O 34		India	Y	21	M
E0973990	O 19		Tunisia	Y	31	M
E0975310	O?		Pakistan	Y	1	M
E0976190	O?		Tunisia	Y	27	M
E0976710	O 57		Tunisia	Y	48	F
E0977730	O?		India	Y	25	M
E0977740	O?		Thailand	Y	24	M
E0977750	O?		Dominica	Y	24	F
E0978430	O?		India	Y	19	F
E0978730	O?		?	Y	<1	M
E0978940	O?		Kenya	Y	25	F
E0979500	O 9		Tunisia	Y	44	F
E0979910	O?		Malta	Y	70	F
E0979920	O?		India	Y	2	F
E0980230	O?		Tunisia	Y	?	F
E0981250	O 9		Tunisia	Y	46	M
E0982340	O?		Turkey	?	?	F
E0983040	O?		Tunisia	Y	45	F
E0983870	O?		India	Y	58	M
E0984680	O?		South America	Y	29	F
E0984690	O?		Tunisia	Y	30	F
E0985930	O?		Thailand	Y	26	M
E0985940	O?		India	Y	21	M
E0987320	O?		Tunisia	Y	52	M
E0987840	O?		Caribbean	Y	47	M
E0988160	O?		India	Y	?	M
E0988840	O?		India	Y	21	F
E0988850	O?		Sri Lanka	Y	32	F
E0989000	O?		Tunisia	Y	44	F
E0989670	O?		Nepal	Y	35	F
E0989680	O?		Sri Lanka	Y	72	F
E0989690	O?		Turkey	Y	28	F
E0989700	O?	fish tank				
E0989940	O?		Malta	Y	53	F
E0989950	O 9		Tunisia	Y	55	F
E0989960	O 9		Tunisia	Y	28	F
E0990720	O?		India	Y	32	F
E099073	O?		?	?	?	F
E0991350	O?		Kenya	Y	24	F
E0991410	O?		?	Y	50	F
E0991900	O 9		Sri Lanka	Y	28	F
E0992030	O 65		Greece	?	65	M
E0992040	O 28		Kenya	Y	52	M
E0992560	O 9	shellfish				
E0993170*	O?	fozen fish				
E0993420	O 11		Tunisia	Y	40	F
E0994200	O?		Kenya	Y	25	F
E0994320	O 9		Malta	Y	51	M
E0994510	O?		India	Y	26	F
E0994920	O?		Turkey	Y	49	F
E0994930	O?		Tunisia	Y	62	M
E0994940	O?		Africa	Y	29	M

Ref.No.	O-group	Source[a]	Country	Diarrhoea[b]	age[c]	sex[d]
E0995570	O?		Tunisia	Y	56	F
E0995580	O?		India	Y	26	F
E0995950	O?		Tunisia	Y	53	F
E0996020	O?		Kenya	Y	52	F
E0996400	O?		Tunisia	?	58	F
E0997480	O?		Turkey	Y	24	F
E0997560	O?		Turkey	?	63	F
E0998200	O?		Dominica	Y	?	M
E0998910	O 9		Dominica	?	58	M
E0998920	O139		Pakistan	Y	33	F
E0999170	O?		Tunisia	Y	35	F
E1000670	O?		Malta	Y	48	F
E1000680	O?		Turkey	Y	54	M
E1000690	O139		India	Y	33	F
E1000950	O?	blood	N. Africa	?	?	M
E1003320	OR		Kenya	Y	64	F
E1004220	O 9		Egypt	Y	26	F
E1004530	O?		Tunisia	?	51	F
E1004790*	O?		India	Y	34	M
E1006290	O?		Thailand	Y	32	F
E1008270	O 7		Kenya	Y	40	F
E1010660	O?		Tenerife	Y	3	M
E1011200	O 65		Indonesia	Y	27	M
E1011480	O 9		Kenya	Y	63	M

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Bengü Said *, H.R. Smith, Sylvia M. Scotland, B. Rowe

Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK

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Detection and differentiation of the gene for toxin co-regulated pili (*tcpA*) in *Vibrio cholerae* non-O1 using the polymerase chain reaction

Bengü Said *, H.R. Smith, Sylvia M. Scotland, B. Rowe

Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK

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Abstract

The polymerase chain reaction has been used to differentiate the gene which encodes the toxin co-regulated pili (*tcpA*) of the El Tor and classical biotypes of *Vibrio cholerae* O1. The same PCR primers were applied to strains belonging to non-O1 serogroups that produced cholera toxin. The size of fragment amplified was either identical to the *tcpA* of biotype El Tor (471 bp) or to the *tcpA* of biotype classical (617 bp). All strains belonging to the novel epidemic serogroup O139 generated a 471-bp fragment identical to El Tor *tcpA*. The present study suggests that there may be an association between non-O1 serogroup and *tcpA* type.

Keywords: Vibrio cholerae non-O1; Toxin co-regulated pilus; Polymerase chain reaction

1. Introduction

Vibrio cholerae serogroup O1, biotypes El Tor and classical, have traditionally been the causative agents of epidemic cholera. *V. cholerae* non-O1 serogroups have been implicated in sporadic cases and outbreaks of gastrointestinal disease which are sometimes clinically indistinguishable from cholera [2,8]. However, there were no recorded epidemics caused by non-O1 serogroups until 1992, when the novel serogroup O139 was described [1,10]. Serogroup O139 has become widespread causing thousands of cholera cases in the Indian subcontinent and adjacent countries. In certain areas O139 appeared to be replacing serogroup O1 as the predominant cause of cholera. However, recent observations indicate epidemics caused by *V. cholerae* O139 are followed by an endemic situation in which *V. cholerae* O1 subsequently reappears [15].

Epidemic O1 and O139 strains produce cholera toxin (CT), which is responsible for the secretory diarrhoea characteristic of cholera. The toxin co-regulated pili (TCP), comprised of a 20.5-kDa pilin subunit called TcpA, are involved in the successful colonization of *V. cholerae* in the small intestine [12]. The TcpA gene, *tcpA*, and the CT genes, *ctxA* and *ctxB*, are coordinately regulated by environmental signals requiring a regulatory protein, ToxR [14]. ToxR is a transmembrane protein which positively regulates these and other virulence factors at the level of transcription. All *V. cholerae* O1 and non-O1, regardless of whether they are toxigenic or non-toxi-

* Corresponding author. Tel.: (+44-81) 200 4400 ext. 3172; Fax: (+44-81) 905 9929

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Table 1
Characterization of *V. cholerae* strains by hybridization with a CT_{A+B} probe and by PCR detection of *ctxB* and *tcpA*

Strain	Source [a]	Country	Serogroup	Biotype	Probe CT_{A+B}	PCR	
						ctxB	*tcpA* [b]
10954/1	TS	Asia	O1 Ogawa	El Tor	+	+	E
8457/5	TS	Asia	O1 Inaba	El Tor	−	−	−
E47021	W	?	O1 Inaba	El Tor	+	+	E
E51116	?	Bangladesh	O1 Ogawa	Classical	+	+	C
E51165	?	Bangladesh	O1 Ogawa	El Tor	+	+	E
N108	W	Australia	O1 Inaba	El Tor	+	+	E
N122	W	Australia	O1 Inaba	El Tor	+	+	E
E90164	?	Mexico	O1 Inaba	El Tor	+	+	E
E90165	?	Mexico	O1 Inaba	El Tor	+	+	E
E46949	D	India	O6		−	−	−
E48065	D	Sri Lanka	O6		−	−	−
E58892	S	Malaysia	O6		−	−	−
N2	W	Australia	O6 [c]		+	+	E
N87	W	Australia	O6 [c]		+	+	E
317-71	TS	Asia	O23		−	−	−
N7	W	Australia	O23 [c]		+	+	C
N9	W	Australia	O23 [c]		+	+	C
N92	W	Australia	O23 [c]		+	+	C
1322-69	TS	Asia	O37		+	+	C
E54380	D	India	O37		−	−	−
E54381	D	Tunisia	O37		−	−	−
E54418	D	Tunisia	O37		−	−	−
1154-74	TS	Asia	O49		−	−	−
E58864	S	S.E. Asia	O49		−	−	−
WBDV-101E	D	Thailand	O49 [d]		+	+	−
E73467	D	India	O49		−	−	−
E85943	D	?	O139		+	+	E
E86270	D	Bangladesh	O139		+	+	E
E87509	D	India	O139		+	+	E
MO45	TS	India	O139 [e]		+	+	E
E87954	D	India	O139		+	+	E
E88131	D	India	O139		+	+	E
E89007	D	India	O139		+	+	E
E90067	D	Pakistan	O139		+	+	E
E90070	D	Pakistan	O139		+	+	E
E90158	D	?	O139		+	+	E
E90159	D	?	O139		+	+	E
E90251	D	Thailand	O139		+	+	E
E55413	D	Egypt	O? [f]		−	−	−
E55879	D	Egypt	O?		+	+	E
E66371	D	Tunisia	O?		+	+	E
E66824	D	Tunisia	O?		−	−	−
CA385	TS	Asia	ORough		+ +	C	

[a] TS, type strain, kindly provided by Dr. R. Sakazaki; W, strain isolated from water; ?, source or country unknown; D, strain isolated from a case of diarrhoea; S, strain isolated from shellfish. [b] *tcpA* PCR results recorded as: C, 617-bp amplicon identical to *tcpA* of the classical biotype; E, 471-bp amplicon identical to *tcpA* of the El Tor biotype; −, negative, no amplicons detected. [c] Strains kindly provided by Dr. P. Desmarchelier. [d] Strain kindly provided by Dr. P. Echeverria. [e] Strain kindly provided by Dr. T. Shimada. [f] O?, untypable with antisera against O1–O83 and O139.

genic, appear to possess *toxR* regulatory sequences. However, all three components, TcpA, CT and ToxR, are essential for *V. cholerae* O1 pathogenesis [6].

A multiplex polymerase chain reaction (PCR) assay targeting *ctxA* and *tcpA* has been used to differentiate biotypes of *V. cholerae* O1 [7]. The oligonucleotide primers targeting *tcpA* exploit sequence differences between the *tcpA* of the El Tor and classical biotypes of *V. cholerae* O1. In the same PCR, *V. cholerae* O139 amplicons aligned exactly with those of El Tor and not with those of classical biotype strains [5]. The O139 serogroup also has the *ctxA* and *tcpA* genes which are coordinately controlled by ToxR [14]. The expression of CT is rare in other serogroups of *V. cholerae* non-O1; in a study which examined wild strains and type strains belonging to serogroups O2 to O83, less than 1% of strains were found to possess *ctxA* or *ctxB* [11]. Our most recent data (unpublished), which includes type strains O2 to O138, have confirmed that CT is uncommon in these serogroups; 12 of 1197 (1%) strains tested are CT probe-positive. In contrast, 87% of 251 strains of *V. cholerae* O1 and all of 13 strains of *V. cholerae* O139 are CT probe-positive.

Production of CT and TcpA by strains belonging to the O139 serogroup has led to speculation that O139 is a variant of O1 biotype El Tor [5]. However, while CT and TcpA have been found in O139 strains, similar studies have not been reported for other *V. cholerae* non-O1. We describe here the characterization of CT-positive strains of *V. cholerae* belonging to O1, O139 and other non-O1 serogroups.

2. Materials and methods

2.1. Bacterial strains

The *V. cholerae* strains used in this study were characterized with respect to the presence of *ctxA* and *ctxB* genes, and for CT antigen production using digoxigenin-labelled polynucleotide probes and an enzyme-linked immunosorbent assay (GM_1-ELISA), as previously described [11]. Strains which hybridized with the CT_A and CT_B probes and CT probe-negative representatives belonging to the same serogroups were used (Table 1). Thirty-four non-O1 strains, of which 22 produced CT antigen, and nine O1 strains, of which eight produced CT antigen, were examined by PCR for *tcpA*. Three of the strains shown in Table 1, 8457/5 (O1), E54418 (O37) and 1154-74 (O49), were CT probe-negative, but GM_1-ELISA-positive [11].

2.2. Polymerase chain reaction (PCR)

For all PCR assays the annealing temperature was 55°C; the reagents and cycling conditions were those described by Fields et al. [4]. A multiplex PCR assay targeting the *tcpA* locus was described by Keasler and Hall [7]. Two sets of primers were used: TCP1 (5′ CAC GAT AAG AAA ACC GGT CAA GAG 3′) and TCP2 (5′ ACC AAA TGC AAC GCC GAA TGG AGC 3′) which amplify a 617-bp fragment of the classical *tcpA*; TCP3 (5′ GAA GAA GTT TGT AAA AGA AGA ACA 3′) and TCP4 (5′ GAA AGG ACC TTC TTT CAC GTT G 3′) which amplify a 471-bp region of El Tor *tcpA*. The presence of *ctxB* was demonstrated using a PCR based on that described by Olsvik et al. [9]. The primers were CTX7 (5′ GGT TGC TTC TCA TCA TCG AAC CAC 3′) and CTX9B (5′ GAT ACA CAT AAT AGA ATT AAG GAT G 3′) which amplify a 460-bp segment of *ctxB*. The three sets of primers were not used simultaneously in the same reaction, because it was difficult to distinguish the 460-bp *ctxB* and the 471-bp El Tor *tcpA* amplicons which co-migrate during electrophoresis.

3. Results

All CT probe-negative strains, including the three strains that were positive in the GM_1-ELISA, were negative by PCR for both *ctxB* and *tcpA*.

The *ctxB* PCR confirmed the probe results (Table 1); all CT probe-positive *V. cholerae*, regardless of serogroup, gave an amplicon of 460 bp (Fig. 1, lanes 6–8).

It was confirmed that the *tcpA* genes of serogroup O1 biotypes El Tor and classical could be differentiated after PCR amplification by amplicon size (Fig. 1, lanes 1 and 2). The non-O1 strains could be similarly differentiated by type of *tcpA* PCR product into two groups (Fig. 1, lanes 3 and 4). The fragments amplified from non-O1 strains were identical

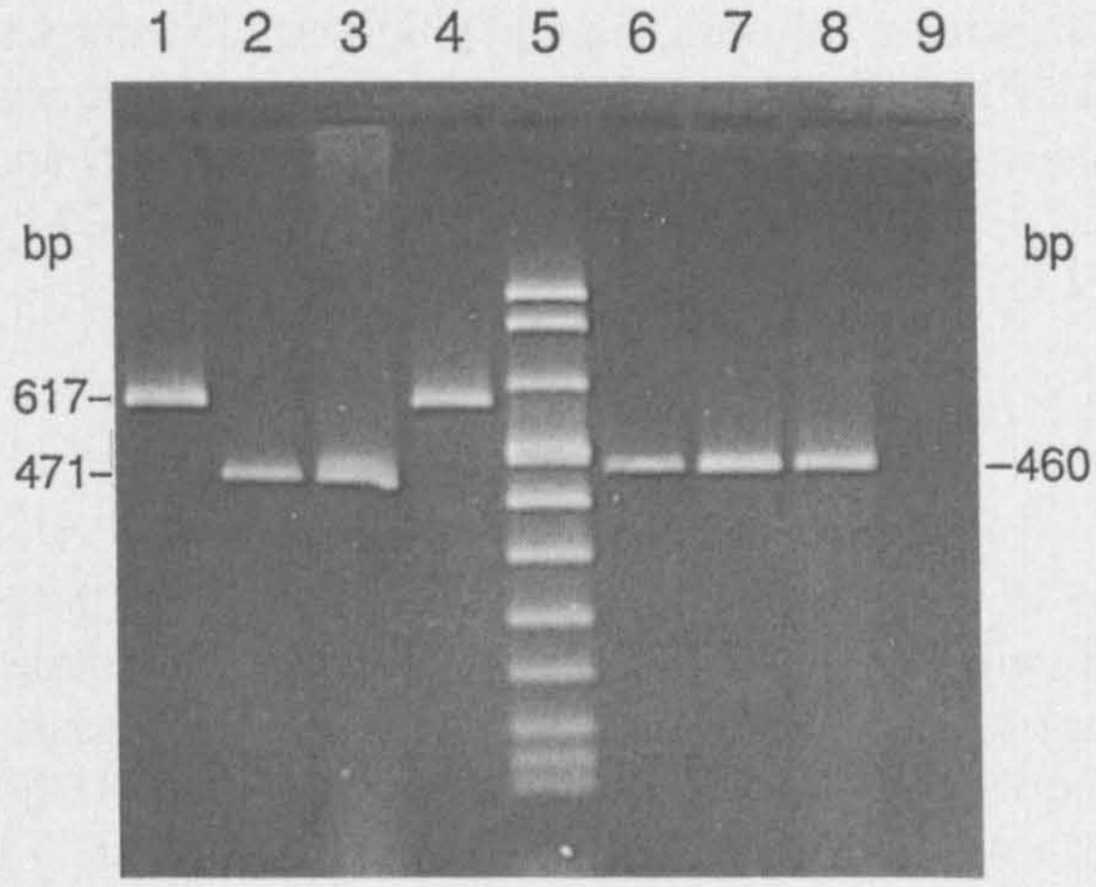

Fig. 1. Differentiation of *V. cholerae tcpA* genes by multiplex PCR and detection of *ctxB* genes. Lanes 1–4 *tcpA* PCR, (1) E51116 O1 classical, (2) E51165 O1 El Tor, (3) MO45 TS O139, (4) 1322-69 TS O37; lane 5, ladder molecular mass markers (bp), 1114, 900, 692, (500, 489), 404, 320, 242, 190, 147, 110, 67; lanes 6–8 *ctxB* PCR, (6) E51116, (7) MO45, (8) 1322-69; lane 9, negative control (sterile glass-distilled water).

in size to the *tcpA* amplicon generated from biotype El Tor or classical. The apparent doublet band in lane 3 of Fig. 1 is thought to be an artefact due to overloading of the gel. Serogroups O23, O37 and a rough strain possessed a *tcpA* identical to the *tcpA* of classical strains, whereas serogroups O6, O139 and two strains with untypable O antigens possessed a *tcpA* identical to El Tor *tcpA* (Table 1). Only one *ctxB*-positive strain, WBDV-101E, belonging to serogroup O49 was negative for *tcpA* by PCR. This strain may either have lost the gene for *tcpA* or it may possess a novel *tcpA* which is sufficiently different from the El Tor and classical *tcpA* to render it undetectable by the primers used in this PCR.

4. Discussion

CT-positive *V. cholerae* non-O1 serogroups produced PCR products which were either identical to those of biotype El Tor or those of classical. All O139 strains tested possessed a *tcpA* identical or similar to El Tor *tcpA* and the present study suggests that a similar association exists between other non-O1 serogroups and *tcpA* type. However, confirmation of this would require testing greater numbers of CT-positive strains belonging to these serogroups and from different geographical origins.

Toxins other than CT have been implicated in the pathogenesis of diarrhoea caused by *V. cholerae* non-O1 [2,8]. CT probe-negative, GM_1-ELISA-positive strains produce a putative toxin which may share epitopes with CT_B [11]. It was postulated that they may possess related gene sequences, but the present study did not support this as three strains tested were negative by PCR for both *ctxB* and *tcpA*.

It has been suggested that strains of serogroup O139 are El Tor variants based on the results of RFLP analysis and on PCR targeting *ctxA* and *tcpA* [5]. In this study we have shown that *ctxB* and *tcpA* gene amplicons of O139, O1 biotype El Tor and certain O6 and O? strains are indistinguishable in size. Rather than suggesting that CT-positive non-O1 are variants of O1, it may be that transfer of CT and TcpA genes has occurred several times from O1 to non-O1 strains and that CT-positive strains of a particular serogroup may be members of a clone resulting from this event.

CT genes are chromosomal and inherited as part of a virulence cassette, along with other factors, including accessory cholera toxin, zonula occludens toxin and TcpA [3,12,13]. There are two CT virulence cassettes which can be differentiated by *tcpA* type and in any one serogroup of *V. cholerae* it appears that only one may be present. Although serogroups other than O1 and O139 have this virulence cassette, to date none have demonstrated epidemic potential. It is interesting to speculate whether there is an as-yet-undiscovered 'epidemic factor'.

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cephalosporins is common among other enteric bacteria.[1,2] The risks associated with the ease for further dissemination of resistance markers in vibrio, as recognised previously,[3,4] and their likely mediation in the recruitment of resistance genes for first-line drugs in cholera treatment deserve close surveillance.

A Rossi, M Galas, N Binztein, M Rivas, M I Caffer, A Corso, M Radice, G Gutkind

Instituto Nacional de Microbiologia "Dr Carlos G Malbrán", Velez Sarsfield 563, Buenos Aires (1281), Argentina; and Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires

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Emergence of multiple drug resistance in *Vibrio cholerae* 01 El Tor from Ecuador

SIR—*Vibrio cholerae* O1 biotype El Tor first appeared in South America in January, 1991, in Peru[1] and by September, 1992, had accounted for over 480 000 documented illnesses and 3000 deaths in that country alone.[2] The disease has rapidly spread across South America at the rate of one country per month and substantial outbreaks have been reported in several countries.[1,2] In Ecuador, strains were first isolated in February, 1991,[1] and outbreaks have subsequently occurred with more than 40 000 people known to be affected.

We have recently received twelve strains of *V cholerae* O1 biotype El Tor isolated in Guayaquil, Ecuador, in 1992. Of these, two strains were resistant to (mg/L) ampicillin (A): 100; chloroamphenicol (C), 125; kanamycin (K), 125; sulphonamides (Su), over 500; tetracyclines (T), 64; and trimethoprim (Tm), over 125 (called resistance-type [R-type] ACKSuTTm). Ten strains were sensitive to all antimicrobial drugs tested. In both resistant strains the complete spectrum of resistance was encoded by a conjugative plasmid of 100 MDa belonging to plasmid incompatibility group C (Inc C). On transfer to *Escherichia coli* K12, a range of exconjugants coding for ACKSuTTm, AKSuTTm, SuTTm, and Su only were identified. The ten drug-sensitive strains were plasmid-free. All twelve strains tested positive with a DNA probe encoding for the A and B subunits of the cholera-toxin gene (CT A + B).

The first outbreak of multiresistant *V cholerae* El Tor occurred in Tanzania in 1977. The causative strain was R-type ACKSSuT (S = streptomycin) and the widespread use of tetracyclines for prophylaxis was thought to have been a major contributory factor.[3] Strains with multiple resistance have subsequently caused outbreaks in the Matlab area of Bangladesh in 1980 and 1981. The 1980 strains were R-type AKSTTm[4] and those in 1981 were R-type AKSuTG (G = gentamicin).[5] In both the African[6] and Bangladesh[3,5] outbreaks, resistances were encoded by 100 MDa Inc C plasmids, and results with the drug-resistant strains from Ecuador confirm the affinity of *V cholerae* O1 for Inc C drug resistance plasmids.

For the treatment of cholera, the WHO Guidelines for Cholera Control recommend doxycycline, a long-acting form of tetracycline, for adults and co-trimoxazole in tablet or liquid formulation for children.[7] Furazolidone, erythromycin, and chloramphenicol are considered to be effective alternatives for adults and children. It is also recommended that the choice of antibiotic should take into account local patterns of resistance.[7] However, rehydration is the therapy of choice. Doctors in South American countries should be aware that strains resistant to three of the drugs of choice for the treatment of cholera have now emerged in Ecuador.

E J Threlfall, B Said, B Rowe

Laboratory of Enteric Pathogens, Central Public Health Laboratory, London NW9 5HT, UK

A Dávalos-Pérez

Department of Clinical Research, Hospital Vozandes, Quito, Ecuador

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Tandem repeats of cholera toxin gene in *Vibrio cholerae* 0139

SIR—In November, 1992, a cholera-like outbreak started in Madras and soon assumed an epidemic proportion in many parts of India[1] and Bangladesh.[2] Tens of thousands of cases were involved with substantial mortality. The causative agent was identified as *Vibrio cholerae*. Serological studies showed these outbreak strains to be similar but distinctly different from known serogroups and were consequently assigned a new serogroup O139.[3] As the clinical manifestations were indistinguishable from those of typical cholera, cholera toxin (CT) was thought to be the major pathogenic attribute. Indeed, all the O139 strains isolated produced CT and hybridised with the CT-gene probe.[4] The counterpart of this organism—namely *V cholerae* O1 (classic and El Tor biotypes)—which were responsible for the past 7 pandemics over 100 years, varied widely in the number of copies of the toxin gene in their chromosomes.[5] We examined the structural characteristics of the toxin operon in *V cholerae* O139 strains isolated from various places.

Advantage was taken of the fact that restriction enzymes *Xba*I and *Bgl*II cleave *V cholerae* O1-derived toxin operons into fragments carrying more than 95% of the toxin gene. Chromosomal DNA prepared from various isolates was digested with *Xba*I and *Bgl*II, the fragments were separated by electrophoresis on agarose gel, transferred to nylon membrane (Hybond-N, Amersham, UK), and hybridised with the CT probe.[6] Complete digestion of the DNA produced two very strong signals, which corresponded to 1·7 kb and 2·5 kb for all strains tested, indicating the presence of at least two copies of the toxin gene (figure, left-hand side). Surprisingly, when digestion was partial, besides the normal 2·5 kb and 1·7 kb bands, a large number of fragments of molecular sizes 18, 12, 9, 6·6, 4·7, 4·3, and 2·2 kb also hybridised with the CT-gene probe. Examination of the pattern revealed that these products of partial digestion could only be generated if the fragments containing CT gene were duplicated many times on the

Additionally, the narrow aperture encouraged sharps to remain vertical and protrude as the bin approached fullness. In comparison, the old bin had a wide letterbox style aperture comprising a shute and flap, which encouraged articles to settle horizontally.

We therefore checked the specifications in BS7320 concerning height of lines indicating that the container is full. The standard states that "a horizontal line to indicate when the sharps container is filled to between 70% and 80% of the maximum volume" should be marked on the container. When we measured the bin it complied with BS7320.

We suggest that the line indicating that the container is full should not be based on a percentage of the volume of the box but instead should be a minimum distance below the aperture, irrespective of the size of the bin. The current British Standard, on the one hand, does not give adequate clearance between the line and the aperture for smaller containers and, on the other, could be providing excessive clearance for the largest containers. Even more critical is the design of the aperture, which ideally should be of the wide letterbox. In our experience, a bin with a narrow aperture is at worst dangerous and at best wasteful of space in the container as it leads to inefficient filling.

We also question how effective it is to have a line marked on the side of a container. Some manufacturers have the line marked only on the front of the bin, although the bin may be turned so that the line is not visible in practice. Also, when sharps containers are placed on clinical trolleys they are often viewed from above, in which case the purpose of the line may be negated. Some indication inside the container that can be viewed through the aperture would help to prevent overfilling.

Injuries from overfilled sharps containers are unlikely to be totally eliminated because of the human element. If simple changes in the design of containers can help reduce their frequency they should be introduced.

M J WEINBREN
R M PERINPANAGAGAM
A HARDWICK

Queen Mary's University Hospital,
London SW15 5PN

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Using condoms to prevent transmission of HIV

EDITOR,—In a study of female prostitutes working in London Helen Ward and colleagues found that the prevalence of HIV infection was less than 1% and that reported regular use of condoms with commercial clients was high (98%).[1] This provides a striking contrast to studies in sub-Saharan Africa. There the prevalence of HIV infection in women regularly engaging in "one off" sexual contacts (including commercial sex workers and bar girls) is often high and regular use of condoms usually low.[2] We have used a published simulation model of the transmission of HIV to examine what effect different levels of use of condoms, in one off contacts only, might have on the spread of HIV infection in rural Uganda.

Using data from the Medical Research Council and Overseas Development Administration's research programme on AIDS in Uganda and the simulation model SimulAIDS (V5.02), we have replicated the spread of HIV infection, and other characteristics, in a rural population in south west Uganda, where the prevalence of HIV infection among adults was about 9% in 1990 (N J Robinson *et al.* IXth international conference on AIDS, Berlin, 7-11 June 1993). For this scenario it was assumed that people did not use condoms. When simulations were rerun from the introduction of HIV, but assuming that condoms were used during 90% of one off sexual contacts, the prevalence of HIV infection among adults in 1990 failed to reach 1%. Furthermore, we found that, even when HIV infection has become widespread, it still seems possible to reduce the future incidence of the infection in the general population substantially by promoting regular use of condoms, even if only in one off sexual contacts (N J Robinson *et al,* IXth international conference on AIDS, Berlin, 7-11 June 1993).

Probably the relatively slow spread of HIV infection among heterosexuals in Britain is at least partly due to widespread regular use of condoms during contacts between sex workers and their clients. Ways must be found to increase use of condoms among people regularly engaging in one off sexual contacts in sub-Saharan Africa. This will benefit not only individual people but also entire populations.

NOAH JAMIE ROBINSON
RICHARD HAYES

Tropical Health Epidemiology Unit,
London School of Hygiene and Tropical Medicine,
London WC1E 7HT

DAAN MULDER

MRC/ODA Research Programme on AIDS in Uganda,
Uganda Virus Research Institute,
Entebbe, Uganda

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Vibrio cholerae serogroup O139 in England and Wales

EDITOR,—Since the beginning of the year there have been several reports from the Indian subcontinent of a large outbreak of cholera caused by non-O1 *Vibrio cholerae.* The outbreak was first reported in Madras last October and was then reported in Calcutta in November[1]; further reports followed from southern Bangladesh in December. An outbreak occurred at a religious festival in Dhaka in January this year and was followed by another major epidemic in Dhaka in the first week of February.[2] A further major outbreak was reported in Calcutta in February.

Although some strains of non-O1 *V cholerae* have been recognised as causing diarrhoeal disease that is sometimes clinically indistinguishable from cholera, previously only *V cholerae* O1 has been considered to be capable of causing epidemic cholera. The strains from the present outbreaks have been placed in a new serogroup, designated *V cholerae* O139 by Shimada *et al.*[3] These strains produce cholera toxin.[1]

In the Laboratory of Enteric Pathogens in London we have been serotyping strains of *V cholerae* since 1985, using the scheme of Sakazaki and Donovan,[4] with antiserum for antigens O1-O83. Since 1985 we have serotyped 1017 isolates. Fifty six were *V cholerae* O1 and 961 were non-O1 *V cholerae.* Serotyping of the 961 isolates of *V cholerae* non-O1 showed that 520 could not be typed with antiserum to O antigens O1-O83. Of these 520 isolates, 51 were from travellers known to have returned recently from the Indian subcontinent and four were from prawns imported from the region. We tested these 55 isolates with antiserum to *V cholera* O139 (provided by Dr T Shimada, National Institute of Health, Tokyo, Japan) and found five to be serogroup O139. We also tested these 55 isolates with DNA probes encoding for the A and B subunits of the cholera toxin gene; only the five isolates of serogroup O139 yielded a positive result. Production of toxin was shown in all five strains.

These five isolates were resistant to streptomycin 16 mg/l, sulphonamides 64 mg/l, and trimethoprim 2 mg/l. They were sensitive to tetracyclines 8 mg/l, ampicillin 8 mg/l, chloramphenicol 8 mg/l, and ciprofloxacin 1 mg/l. Sarkar *et al* reported that the epidemic strains of *V cholerae* non-O1 that they had received from different locations in India were all sensitive to polymyxin B (15 mg/l and 50 mg/l).[5] We tested the five strains of *V cholerae* O139 isolated in England and also the type strain of *V cholerae* O139 (MO45) provided by Dr T Shimada and found that all six strains were resistant to polymyxin B, with minimum inhibitory concentrations $>2{\cdot}5\times10^4$ U/l.

The five strains of *V cholerae* O139 were all isolated in England since March this year (from four adults and one child). They were isolated from three travellers who had recently returned from India, one who had recently returned from Bangladesh, and one from whose records we were unable to establish a history of recent travel. All five patients had diarrhoea, and three of them (two adults, one child) were admitted to hospital.

V cholerae serogroup O139 is unlikely to pose a serious threat in countries with safe drinking water and good sanitation. Nevertheless, the severity of the disease caused by this serogroup means that medical microbiologists should be aware of the potential importance of non-O1 *V cholerae* in diarrhoeal disease and should refer all isolates of non-O1 *V cholerae* to the reference laboratory for confirmation, serotyping, and testing for toxins.

T CHEASTY B ROWE
B SAID J FROST

Laboratory of Enteric Pathogens,
Central Public Health Laboratory,
London NW9 5HT

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Therapeutic potential of aspirin in cancer of the colon

EDITOR,—R F A Logan and colleagues' epidemiological study supports the growing evidence that regular use of aspirin reduces the risk of colorectal cancer.[1] Annlia Paganini-Hill, however, advocates caution before prophylactic aspirin is considered, since long term use of aspirin is associated with nephrotoxic and gastrotoxic side effects.[2] But other valuable lessons can be learnt from the epidemiological studies. We wish to propose an explanation for the epidemiological data and suggest further directions for research.

Aspirin and other non-steroidal anti-inflammatory drugs inhibit and reverse the growth of tumours of the colon in animals. This effect correlates with the inhibition of prostaglandin E_2,[3] a derivative of fatty acid that is produced in excessive quantities by these tumours. Prostaglandin E_2 derived from tumour seems to further growth in the colon by depressing cellular immunity, enhancing local blood flow, and increasing metastatic potential. By reversing these pathophysiological processes non-steroidal anti-inflam-

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The use of gene probes, immunoassays and tissue culture for the detection of toxin in *Vibrio cholerae* non-O1

BENGÜ SAID, SYLVIA M. SCOTLAND and B. ROWE

Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

Summary. *Vibrio cholerae* non-O1 strains were screened for the presence of cholera enterotoxin (CT) genes by means of digoxigenin-labelled polynucleotide CT_A and CT_B probes. In-vitro production of CT was investigated by the Y1 mouse adrenal cell assay, enzyme-linked immunosorbent assay (ELISA) and a commercial, reversed passive latex agglutination (RPLA) kit. Only two (0·25%) of 790 strains tested gave positive results with the CT_A and CT_B probes. The production of other bacterial cytotoxin(s) made it impossible to use the characteristic cell-rounding effect on Y1 cells for the detection of CT. CT production by the probe-positive strains was confirmed by the immunoassays. Two hundred and fifty-two of the 788 probe-negative strains were tested by both cell assay and immunoassays. Of these, 90% produced cytotoxin(s) in the cell assay. In addition, 37% gave positive results in CT-ELISA, but negative results with LT-ELISA and VET-RPLA. These results indicate the presumed presence of a toxin in *V. cholerae* non-O1 that is able to bind GM_1 and react with antisera to CT, but which is not identical to CT.

Introduction

Vibrio cholerae can be distinguished serologically on the basis of somatic (O) antigens. There are currently 82 known serogroups in addition to *V. cholerae* O1, the causative agent of epidemic cholera.[1] *V. cholerae* non-O1 strains are distributed world-wide, often in brackish surface waters. Some serogroups have been implicated in sporadic cases and outbreaks of gastrointestinal disease. The symptoms vary from bloody diarrhoea with fever to a severe watery diarrhoea indistinguishable from cholera.[2]

Cholera enterotoxin (CT) is a recognised virulence factor of *V. cholerae* O1 and a heat-labile enterotoxin identical or similar to CT has been found in some isolates of *V. cholerae* non-O1.[3,4]

CT production in strains of *V. cholerae* non-O1 has been reported from both Bangladesh and India; Datta-Roy[5] found 26% of a small sample of 34 clinical isolates and 10% of environmental isolates to give positive results in tests for CT production. However, the occurrence rate of CT in *V. cholerae* non-O1 in other parts of the world is lower. None of 44 human isolates and only 2% of environmental isolates from Thailand were reported to be CT positive.[6] Similarly, in a study of 2500 environmental isolates from the Louisiana Gulf coast only 0·3% produced CT.[7] However, in none of these studies have the serogroups of *V. cholerae* non-O1 been reported, nor has a correlation of CT production with serogroup been attempted.

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The somatic antigens may be useful indicators of diarrhoeagenic potential within the non-O1 strains. In 1980, the WHO working group[8] reported a prevalence of serogroups O5 in gastrointestinal disease and O8 in the environment, whereas Donovan[9] found that serogroups, O2, O5, O7 and O37 predominated in gastrointestinal disease and O4 in the environment. Certain serogroups have also been linked to outbreaks of gastrointestinal illness: e.g., serogroup O5 is thought to have been responsible for a food-borne outbreak in Czechoslovakia in 1965[10] and serogroup O37 for a water-borne outbreak in the Sudan in 1968.[11] Recent reports from India and Bangladesh have described large outbreaks due to CT-producing *V. cholerae* non-O1.[12,13] The strains from India were untypable (O?) and, although serogrouping for the Bangladesh outbreak remains to be done, both outbreaks may have been caused by strains from the same clone.

The Laboratory of Enteric Pathogens (LEP) has a large collection of *V. cholerae* non-O1, belonging to many different serogroups, isolated from over 50 countries. To assess the correlation of CT with serogroup, strains from both environmental and human sources were examined for the presence of the CT genes and for the production of heat-labile enterotoxins with the Y1 cell test and immunoassays.

Materials and methods

Bacterial strains

Seven hundred and eight wild-type strains of

V. cholerae non-O1, from both human and environmental sources, were studied; all strains were taken from the culture collection of LEP. Of these, 310 strains belonged to 49 serogroups, 334 strains were untypable (O?) and 64 strains were rough (OR). The type strains of *V. cholerae* non-O1 (serogroups O2–O83) were kindly provided by Dr R. Sakazaki. The *V. cholerae* non-O1 control strain WBDV-101E (serovar O49, CT) was kindly provided by Dr P. Echeverria. The *V. cholerae* O1 control strain (E51116, CT) came from the LEP culture collection. All *V. cholerae* strains were maintained on nutrient-agar slopes at room temperature. The control *Escherichia coli* strain B7A (O148:H28), which produces both heat-labile enterotoxin and heat-stable enterotoxin, (LT^+ STa^+)[14] was maintained on a Dorset's egg slope at room temperature.

Preparation and digoxigenin-labelling of polynucleotide CT_A and CT_B probes

Strains of *E. coli* K12 carrying plasmids with cloned CT gene sequences were used to prepare large quantities of covalently closed circular DNA.[15] The CT gene sequence from *V. cholerae* El Tor 62746[16] cloned into plasmid pBR325 (pCVD27) was provided by Dr J. B. Kaper. A 554-bp CT_A probe was cut from pCVD27 after digestion with *Eco*RI (Life Technologies Ltd, Uxbridge, Middlesex). The recombinant plasmid pCT19 is known to contain CT_B and part of CT_A;[17] the gene sequences were cloned and subcloned from *V. cholerae* 1621 into pACYC184 as described by Gennaro *et al.*[18] A 550-bp CT_B probe was cut from plasmid pCT19 after digestion with *Xba*I and *Hin*cII. Probe fragments were separated on and excised from agarose gels and purified with the Geneclean Bio 101 kit (Stratech Scientific Ltd, Luton, Bedfordshire). The cloned polynucleotide probes were labelled with digoxigenin according to the manufacturer's instructions (Boehringer Corporation Ltd, Lewes, East Sussex). Unincorporated nucleotides were removed with QIAGEN-tip 5 (Diagen, Dusseldorf, Germany) according to the manufacturer's instructions and the labelled probe was stored at −20°C.

Preparation of cultures for DNA hybridisation

Bacterial cultures, grown at 37°C with aeration for 18 h, were spotted on to Hybond N nylon membranes (Amersham International, Aylesbury, Buckinghamshire) placed on nutrient agar plates. Colonies were grown at 37°C for 6 h and the cells were lysed and DNA denatured as described by Maniatis *et al.*[15]

Hybridisation with polynucleotide probes

Hybridisations were performed in heat-sealed plastic bags which were placed in a water bath at the appropriate temperature with shaking. Hybridisation was carried out at 68°C (high stringency conditions identifying sequences with > 80% identity) on colony blot membranes. In some experiments, lower stringency conditions (37°C) were used, allowing the hybridisation of sequences with only 60% identity.

Colony blot membranes were pre-washed with 10 ml of a hybridisation solution per 50 cm^2 membrane for 1 h at 68°C. The hybridisation fluid contains; five times concentrated SSC (where SSC consists of NaCl 0·88%, trisodium citrate 0·44%), blocking reagent (Boehringer) 0·5%, N-lauroylsarcosine 0·1%, SDS 0·02%.) The membranes were wiped and pre-hybridised with fresh solution for 2 h. Hybridisation was allowed to proceed for 18 h, with hybridisation solution 1·25 ml/50 cm^2 membrane, containing freshly denatured probe 26 ng/ml and freshly denatured herring sperm DNA 100 μg/ml. Membranes were washed with double strength SSC, SDS 0·1% for 5 min at room temperature followed by two washes of 15 min each of, tenth dilution SSC, SDS 0·1% at 68°C. Detection procedures were as described by Boehringer.

The protocol for hybridisation at 37°C did not differ from that described above, but the hybridisation solution was formamide 25%, five times concentrated SSC, Ficoll 0·01%, polyvinylpyrrolidine 0·01%, bovine serum albumin 0·01%, SDS 0·1%, and 1 mM EDTA. The post-hybridisation washes were with five times concentrated SSC, SDS 0·1% at 54·5°C for 1 h, followed by double strength SSC at room temperature for 30 s.

Bacterial culture and preparation of crude toxin

For routine testing of CT production by *V. cholerae* O1, bacteria were grown overnight in Syncase sucrose broth[19] at 37°C with agitation. Initially these conditions were used to test *V. cholerae* non-O1 strains. For further assays of CT production, culture filtrates were prepared by growing the organisms in various media: Syncase sucrose broth, Syncase glucose broth,[19] Trypticase Soy Broth (Becton Dickinson, Cockeysville, USA),[20] Brain Heart Infusion Broth (Unipath Ltd, Basingstoke, Hampshire),[21] and casamino yeast extract broth.[22,23] Strains were inoculated into 10-ml volumes of the respective broths and incubated statically in a 250-ml flask at 30°C and 37°C, or with agitation (120 oscillations/min). The overnight culture was then centrifuged (17000 *g* for 30 min at 4°C) and the supernate was sterilised by filtration through a Millipore filter (pore size 0·2 μm). A sample of the culture supernate was also heated at 100°C for 15 min. The filtrate and the heated sample were used for both tissue culture tests and immunoassays. All tests were done in duplicate.

Y1 adrenal cell assay for toxin

Culture filtrates were tested for CT on monolayers of Y1 cells.[21,24] CT and *E. coli* LT characteristically lead to a rounding of the Y1 cells. The effect of the

heated preparation was compared with that of the unheated to ensure that rounding was due to a heat-labile factor. Doubling dilutions of filtrate were used to determine titres of heat-labile enterotoxin and cytotoxin. Neutralisation with antiserum against CT or LT was performed to confirm the specificity of the rounding effect. For the neutralisations, 25-μl volumes of filtrate and antiserum (1 in 200) were incubated together in 200 μl of growth medium for 3 h at 37°C before being transferred on to Y1 cells.

Enzyme-linked immunosorbent assay

A modification of the GM_1-ELISA methods of Svennerholm and Holmgren[25] and Sack[26] was used. A solution of ganglioside GM_1 (2 μg/ml Supelco, Bellefonte, USA) 100 μl in PBS was added to micro-ELISA plates (Dynatech Laboratories Ltd, Billingshurst, Sussex) and incubated overnight at room temperature. Extra binding sites were blocked by adding 200 μl of BSA (Sigma, Poole, Dorset) 1% w/v dissolved in PBS, for 30 min at 37°C. The steps of the procedure were as follows. After each step the plates were washed three times with PBS containing Tween 20 (PBST) 0·05% v/v; 100 μl of the filtrate were added and the plates were incubated at room temperature for 2 h. One hundred μl of the appropriate antiserum, diluted 1 in 200 in PBS were added: for CT-ELISA, rabbit anti-CT antiserum (LEP); for CT_B-ELISA, goat anti-CT_B antiserum (Calbiochem Novabiochem, Nottingham); for LT-ELISA, rabbit anti-LT antiserum (LEP). The plates were then incubated for 18 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma) 100 μl or alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin (Sigma) 100 μl were added. Antibody preparations were diluted 1 in 7000 and 1 in 3000 in PBS respectively. The plates were incubated at room temperature for 2 h. Then 200 μl of p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer) were added. After 100 min in the dark at room temperature, the reaction was stopped with 25 μl of 1 M NaOH and the absorbance was read at 405 nm. A control (strain E51116) was included on each plate.

Reversed passive latex agglutination

The VET-RPLA kit (Unipath Ltd) was used for the detection of CT and LT. Doubling dilutions of filtered supernates were tested according to the manufacturer's protocol.

Results

CT_A and CT_B probes at high stringency

The control strains (WBDV-101E, E51116 and B7A) and 790 *V. cholerae* non-O1 strains were tested by hybridisation at high stringency with polynucleotide probes derived from both the CT_A and CT_B regions of the CT gene sequence. Strains WBDV-101E (serogroup O49) and E51116 hybridised with both the CT_A and CT_B probes. Strain B7A did not hybridise under high stringency conditions.

Only two of the 790 *V. cholerae* non-O1 strains were probe-positive. These were: a wild-type strain (E55879) serogroup O?, isolated from a 22-year-old male recently returned to the UK from Egypt; and strain 1322-69, the type strain of serogroup O37.

Of the 788 probe-negative *V. cholerae* non-O1 strains, five were serogroup O49 (all from environmental sources), four were O? (from patients returning from Egypt) and seven were serogroup O37 (all from human sources). The remaining typable strains belonged to 47 different serogroups.

CT_A and CT_B probes at low stringency

The heat-labile enterotoxin produced by *E. coli* is known to be related to CT.[27] However, in this study, hybridisation experiments showed that LT^+ *E. coli* hybridised with the CT probes only at low stringency. *V. cholerae* non-O1 strains were also tested for hybridisation at low stringency with the CT_A and CT_B probes to determine if a CT/LT-related toxin was being produced. A total of 252 strains (representing 37 different serogroups from both human and environmental sources) was tested and all were probe-negative under low stringency conditions.

Y1 mouse adrenal cell assay

In all, 226 (89·6%) of 252 *V. cholerae* non-O1 strains tested in the Y1 cell assay produced a cytotoxic effect on Y1 cells within 24 h; the remaining 26 strains were completely negative. Of the identifiable serogroups producing cytotoxin, the most common were: O26 (23 of 26 strains tested were cytotoxin positive), O2 (10 of 10), O19 (9 of 9), O5 (7 of 7), O13 (4 of 4), O14 (4 of 4), O49 (4 of 4), O76 (4 of 4) and O37 (3 of 3). It is noteworthy that all strains from serogroups O2, O5, O13 and O37 produced cytotoxin, because these serogroups have been reported to be associated with human disease.[8,9] The probe-positive *V. cholerae* non-O1 strains also produced cytotoxin(s) which made it impossible to distinguish true rounding from early cytotoxic effects. Even when filtrates were diluted to 1 in 156250, cytotoxin was still present. The cytotoxic effect present in the filtrates, even as CT, was destroyed by heating at 100°C for 15 min, but the effect was not neutralised by antiserum to cholera toxin. The control *V. cholerae* O1 strain did not produce cytotoxin under these conditions, but did cause typical rounding of the cells which was confirmed as CT by neutralisation. Similarly, *E. coli* strain B7A produced rounding typical of LT and this effect could be neutralised with anti-cholera antiserum.

The probe-positive *V. cholerae* strains and *E. coli* strain B7A were tested for CT (or LT) production

Table. CT production under different growth conditions

Culture medium	Aeration/ temperature (°C)	ELISA* and VET-RPLA†					Y1 Cell Test‡				
		E55879 O?	1322-69 O37	WBDV-101E O49	E51116 O1	B7A *Escherichia coli*	E55879 O?	1322-69 O87	WBDV-69 O49	E51116 O1	B7A *E. coli*
Syncase sucrose broth	+/37	+(32)	+(64)	+(64)	+(32)	+(128)	c	c	c	2560	156250
	−/37	+(8)	+(4)	+(8)	+(4)	+(32)	c	c	c	40	6250
	+/30	+(16)	+(32)	+(32)	+(32)	+(16)	c	c	c	1280	250
	−/30	+(4)	+(4)	+(4)	+(4)	+(16)	c	c	c	40	250
Syncase glucose broth	+/37	+(ND)	−(ND)	+(ND)	+(ND)	+(ND)	c	—	c	40	6250
	−/37	−(ND)	−(ND)	−(ND)	+(ND)	+(ND)	—	c	c	40	31250
	+/30	+(ND)	+(ND)	+(ND)	+(ND)	+(ND)	c	c	c	1280	250
	−/30	+(ND)	+(ND)	+(ND)	+(ND)	+(ND)	c	c	c	320	1250
Trypticase soy broth	+/37	+(32)	−(0)	+(8)	+(32)	+(128)	c	c	c	160	156250
	−/37	−(0)	−(0)	−(0)	+(32)	−(0)	—	c	—	320	250
	+/30	+(16)	+(4)	+(4)	+(16)	+(16)	c	c	c	c	1250
	−/30	+(8)	−(0)	−(0)	+(8)	−(0)	c	c	—	40	250
Brain heart infusion broth	+/37	+(ND)	−(ND)	+(ND)	−(ND)	+(ND)	c	c	c	20	6250
	−/37	+(ND)	−(ND)	−(ND)	−(ND)	+(ND)	c	c	—	—	1250
	+/30	+(ND)	−(ND)	+(ND)	+(ND)	−(ND)	c	c	c	40	250
	−/30	+(ND)	−(ND)	+(ND)	+(ND)	+(ND)	c	c	c	40	250
Casamino yeast extract broth	+/37	+(8)	+(8)	+(16)	+(16)	+(32)	c	c	c	160	31250
	−/37	+(4)	+(2)	+(4)	+(8)	+(32)	c	c	c	40	6250
	+/30	+(8)	+(8)	+(8)	+(8)	+(4)	c	c	c	80	250
	−/30	+(2)	+(2)	+(4)	+(2)	+(4)	c	c	c	20	250

ND, not done; c, cytotoxin.
* ELISA positive OD 405 nm > 1.
† Figure in parenthesis is titre of reversed passive latex agglutination for CT and LT.
‡ The final titre is shown for cytotonic response.

under various growth conditions (table). For the non-O1 strains, none of the conditions allowed the differentiation of CT from the cytotoxin in the Y1 test. For strain E51116, the highest CT titre (2560) was obtained after overnight incubation with agitation at 30°C in Syncase sucrose broth. *E. coli* B7A gave the highest titre (156250) after overnight incubation at 37°C with agitation with either Syncase sucrose broth or TSB.

ELISA

The probe-positive *V. cholerae* non-O1 strains, *E51116 and B7A were tested in the CT, CT_B and LT-*ELISA assays. All gave positive results in these three ELISAs.

Ninety-three (36·9%) of 252 probe-negative *V. cholerae* non-O1 strains tested in the CT-ELISA gave a positive result ($OD_{405} > 1$). These 93 CT-ELISA positive strains also gave positive results in the CT_B-ELISA, but all gave negative results in the LT-ELISA. Fifty-six (60%) of these 93 strains could not be serogrouped. The commonest of the identifiable serogroups were O26 (eight strains) and O19 (seven strains); the remaining 22 strains belonged to 17 other serogroups.

VET-RPLA

The 93 *V. cholerae* non-O1 strains that gave positive results in the CT-ELISA all gave negative results in the VET-RPLA.

The probe-positive strains E55879, 1322-69, E51116 and B7A all gave positive results in this immunoassay. The effect of growth conditions on CT production by the probe-positive strains could be detected easily and quantified in this immunoassay; there were conditions under which no toxin production could be detected by this immunoassay or by the ELISAs. CT production was optimal in Syncase sucrose broth and casamino yeast extract broth. The highest toxin titres—32 for strain E55879 (O?) and 64 for both type strain 1322-69 (O37) and control strain WBDV-101E (O49)—were obtained with Syncase sucrose broth at 37°C with aeration.

Discussion

In this study, V. cholerae non-O1 strains from 50 countries, isolated from both environmental and human sources, were tested for the production of CT by four methods.

Of the *V. cholerae* non-O1 strains which were tested with the CT probes, only three gave positive results, one belonging to serogroup O49 (this was the control strain WBDV-101E), one to O37 and one O? strain (from Egypt). There was no apparent correlation of serogroup with CT production. The low occurrence of CT probe-positive *V. cholerae* non-O1 strains (0·25%) is in agreement with previous reports.[6,7]

CT and LT are part of a heterogeneous family of enterotoxins and different forms of LT have been described; i.e., LTh-I, LTp-I, LTIIa and LTIIb.[28] The suggestion has been made that there may be differences in molecular structure between enterotoxins produced by different serovars of *V. cholerae*.[29] In the present study, hybridisation at low stringency with CT_A and CT_B probes provided no evidence for genes in *V. cholerae* non-O1 encoding heat-labile enterotoxins related to CT or LT. As CT_A is the more conserved part of the toxin in the CT/LT family of toxins[30] it was

considered possible that some strains might hybridise with the CT_A probe only. However, no such strain was detected in this study.

All probe-positive strains were confirmed as CT-producers by immunoassays. However, the CT-ELISA and CT_B-ELISA also gave positive results with some strains which were negative with the CT probes. The ELISA assay depends on the ability of the GM_1 ganglioside to bind heat-labile enterotoxins, with bound toxin detected by specific antitoxin antibodies. The strains that gave positive CT-ELISA results may be producing a heat-labile enterotoxin distinct from CT,[23,31,32] but nevertheless able to bind to ganglioside GM_1. This presumed toxin also appeared to share epitopes with CT, and more specifically with CT_B. Because of the production of cytotoxin, which masks cytotonic effects on Y1 cells, it was not possible to show neutralisation of the putative toxin.

All CT-ELISA-positive strains produced cytotoxin in the Y1 cell assay. However, the CT-ELISA did not detect the cytotoxin, since most strains that gave negative results in the CT-ELISA produced cytotoxin on Y1 cells. Cytotoxin production may be important for virulence, as representatives of all the serogroups associated with diarrhoeal illness (O2, O5, O13 and O37) were found to produce cytotoxin.

The production of CT by *V. cholerae* O1 and LT by *E. coli* strain B7A was detected readily by the conventional Y1 cell assay and immunoassays. The probe-positive *V. cholerae* non-O1 strains produced cytotoxin which effectively masked the CT rounding in the Y1 cell assay and varying the growth conditions did not affect the result. The immunoassays used could detect the presence of CT even in the presence of cytotoxin. The optimal condition for CT production, as detected by immunoassays, was growth in Syncase sucrose broth at 37°C with agitation.

Recent volunteer studies showed that some *V. cholerae* non-O1 strains, which did not produce CT, caused diarrhoea of a similar severity to that seen in cholera.[33] This suggests that factors other than CT, such as the presumed toxin identified in this study, may be important in the pathogenesis of diarrhoea caused by *V. cholerae* non-O1 strains.

In the present study, < 1% of *V. cholerae* non-O1 strains produced CT immunologically indistinguishable from the CT produced by serogroup O1. Because these few strains also produced cytotoxins, CT could be detected only by probe, or by immunoassay under specific growth conditions. Of the probe-negative strains, most (90%) produced cytotoxin(s) detected by Y1 cells. In addition, 37% of probe-negative strains gave positive results in the ELISA for CT but negative results in the ELISA for *E. coli* LT and VET-RPLA. The substance produced was distinct from the cytotoxin(s) detected in the Y1 cell assay. This substance could be detected only in the CT-ELISAs and may be a toxin structurally and antigenically similar to CT. The role in diarrhoeal disease of both the cytotoxin and the substance detected by the CT-ELISA needs to be evaluated.

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